

Prolamin degradation in sourdoughs

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PROLAMIN DEGRADATION IN SOURDOUGHS

JUSSI LOPONEN

ACADEMIC DISSERTATION

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ABSTRACT

This thesis examines protein behaviours that occur during cereal fermentations. The focus is on the prolamin degradation in sourdoughs. The thesis also looks at what happens to the oat globulins during an oat bran acidification process.

The cereal prolamins are unique proteins in many respects. The wheat prolamins (glutenins and gliadins) are responsible for the formation of the gluten that provides the viscoelastic properties to wheat doughs whereas the rye prolamins (secalins) are unable to develop gluten-like structures. In addition, many baking technological features, such as flavour, shelf-life and dough properties are affected by the protein degradation that might occur during processing. On the other hand, the prolamins contain protein structures that are harmful to gluten sensitive people. It is thus evident that the degradation of the prolamins in sourdough processes may be approached from various aspects. This thesis describes some of these approaches.

Four different cereal fermentations were carried out. Wheat sourdough (WSD) and rye sourdough (RSD) fermentations represented traditional sourdoughs. A germinated-wheat sourdough (GWSD) was a novel sourdough type that was prepared using germinated wheat grains that had high and diverse proteolytic activities. The oat bran fermentation (OBF) represented a fermentation system that lacked functional cereal proteases.

The high molecular weight glutenins and rye secalins were degraded during the WSD and RSD fermentations, respectively. It was noteworthy that in WSD only a very limited degradation of the gliadins occurred. The gliadins were, however, hydrolysed very extensively during the GWSD fermentation. No protein degradation was observable in the OBF system. Instead the acidification altered the solubility of the oat globulins and this finally led to their aggregation.

This thesis confirms that the endogenous proteases of cereals hydrolyse cereal prolamins in sourdoughs. The thesis also shows that the proteolytic activity of the used cereal raw material determines the extent of proteolysis that occurs in sourdough. This means that bakers may adjust the protein degradation in their sourdoughs by selecting the raw material based on its proteolytic activity. The thesis also demonstrates that by using germinated grains, with high and diverse proteolytic activity in sourdough preparations, the prolamins can be extensively degraded. Whether such highly proteolytic food technology could be used to manufacture new gluten-free cereal-based products for gluten sensitive people remains to be solved.

PREFACE

This study was carried out in the Cereal Technology Group at the Department of Food Technology, University of Helsinki during the years 2002-2006. The greatly appreciated funding for the study came from several sources: TEKES (the Finnish Funding Agency for Technology and Innovation), ABS Graduate School (the Finnish Graduate School on Applied Bioscience: Bioengineering, Food & Nutrition, Environment), the Finnish Food Research Foundation, and the University of Helsinki Funds.

I am very grateful to my supervisors, Professor Hannu Salovaara and University Lecturer Tuula Sontag-Strohm, for providing me with working facilities and for encouraging me throughout my working period. I also thank Dr. Markku Mikola for introducing me to the world of cereal proteases at the beginning of the study and for showing his interest throughout it. Hannu, Tuula and Markku, it has been a great pleasure to have the opportunity to combine your knowledge on sourdoughs, cereal proteins and cereal proteases in this thesis work.

I am very grateful to the pre-examiners of the thesis, Dr. Berne Jones and Dr. Michael Gänzle, for their constructive criticism. I especially thank Dr. Berne Jones for helping with the English grammar. It appeared that Dr. Michael Gänzle also played a big role in the reviewing processes of the original papers. Thus, I also thank him for his constructive comments at that time.

I thank Kristiina Tuukkanen for the solid coworking we have always had. I am grateful to Dr. Kati Katina and Dr. Jarkko Venäläinen for the nice collaboration. I also thank Lauri Simonson, Kristiina Tuukkanen, Pia Laine, Sari Jantunen, Nina Waldvogel, and Päivi Kanerva for sharing, one by one, the workroom with me during my working period. Especially I thank Kristiina Tuukkanen, Heli Anttila, Päivi Kanerva and Fred Gates for the time they shared with me over the years.

I also thank my friends for the many ways they have supported my work, for instance, for organising the NSD events. To my mother, I am very grateful for all of her support. And to my lovely daughters, Emma and Essi, I owe you zillion hugs for bringing me back to earth daily. Finally, and most importantly, to my wife Jaana, dearest thanks for your support in everything.

Thank you!

Helsinki, November 2006

Jussi Lopenen

LIST OF ORIGINAL PUBLICATIONS AND AUTHORSHIPS

I Loponen J., Mikola M., Katina K., Sontag-Strohm T. and Salovaara H. 2004. Degradation of HMW glutenins during wheat sourdough fermentations. Cereal Chemistry 81:87-93.

The study plan was designed by Jussi Loponen, Markku Mikola, Tuula Sontag-Strohm and Hannu Salovaara. Most of the experimental work and preparation of the manuscript were carried out by Jussi Loponen. Kati Katina was responsible for the preparation of the pure culture sourdoughs and the description of this part in the *Materials and Methods*. Markku Mikola, Tuula Sontag-Strohm and Hannu Salovaara made comments and suggestions during the manuscript preparation. Jussi Loponen was the corresponding author.

II Tuukkanen K., Loponen J., Mikola M., Sontag-Strohm T. and Salovaara H. 2005. Degradation of secalins during rye sourdough fermentation. Cereal Chemistry 82:677-682.

The study design was planned by all of the authors. Kristiina Tuukkanen prepared the first version of the manuscript and carried out most of the experimental studies, after which she left on maternity leave. Jussi Loponen continued the work and carried out the antibiotic sourdough preparations and their analyses and also the proteinase studies. Jussi Loponen also prepared the final version of the manuscript and was the corresponding author. The study supervisors, Markku Mikola, Tuula Sontag-Strohm and Hannu Salovaara participated in the manuscript preparation by giving their comments and suggestions. Kristiina Tuukkanen and Jussi Loponen were equal first authors.

III Loponen J., Sontag-Strohm T., Venäläinen J. and Salovaara H. Prolamin hydrolysis in wheat sourdoughs with differing proteolytic activities. (revised manuscript submitted to Journal of Agricultural and Food Chemistry on November 24th 2006).

The study was designed by Jussi Loponen, Tuula Sontag-Strohm and Hannu Salovaara. Jussi Loponen carried out most of the experimental studies and prepared the manuscript. Jarkko Venäläinen was responsible for the determination of POP and DPP IV activities and also for the description of this work in the *Materials and Methods*. Tuula Sontag-Strohm and Hannu Salovaara made comments and suggestions during the manuscript preparation. Jussi Loponen was the corresponding author.

IV Loponen J., Laine P., Sontag-Strohm T. and Salovaara H. (in press). Behaviour of oat globulins in lactic acid fermentation of oat bran. European Food Research and Technology. (DOI 10.1007/s00217-006-0387-9).

The study was designed by all of the authors. Jussi Loponen carried out most of the experimental part whereas Pia Laine carried out the oat globulin assay and part of the preliminary experiments. Jussi Loponen was responsible for the manuscript preparation and was the corresponding author. Pia Laine, Tuula Sontag-Strohm and Hannu Salovaara provided comments and suggestions during the manuscript preparation.

ABBREVIATIONS

ABI	antibiotic buffered incubation
ACE	angiotensin-converting enzyme
BBM	brush border membrane
CD	celiac disease
CSD	control wheat sourdough
DFP	diisopropyl fluorophosphate (a serine protease inhibitor)
DPP IV	dipeptidyl peptidase IV
DY	dough yield
E-64	epoxysuccinyl-L-leucylamido-(4-guanidino) butane (a cysteine protease inhibitor)
ELISA	enzyme-linked immunosorbent assay
FAN	free amino nitrogen
GI	gastrointestinal
GR	glutathione reductase
GSH	glutathione (reduced, active form)
GSSG	glutathione (oxidised, inactive form)
GWSD	germinated-wheat sourdough
HLA	human leucocyte antigen
HMW	high molecular weight
ISD	inactive wheat sourdough-like ferment
LAB	lactic acid bacteria
LMW	low molecular weight
M _r	molecular weight
NTR	NADPH-dependent thioredoxin reductase
OBF	oat bran fermentation
O-FEN	1,10-phenanthroline (a metalloprotease inhibitor)
PEP	prolyl endoprotease
PEP-A	pepstatin A (an aspartic protease inhibitor)
PMSF	phenylmethylsulphonyl fluoride (a serine protease inhibitor)
POP	prolyl oligopeptidase
RSD	rye sourdough
SDS-PAGE	sodium-dodecyl sulphate polyacrylamide gel electrophoresis
SDS-SB	SDS-PAGE sample buffer
tTGase	tissue transglutaminase
WSD	wheat sourdough

1 INTRODUCTION

Sourdoughs are used in baking to improve the technological, economic and nutritional properties of bread. Sourdough is a rather complex biochemical system that at its simplest comprises flour, starter and water. This means that the biochemical features of the cereals and starter microbes are also included and these may become activated in the semi-fluid sourdough system. This thesis focuses on the cereal protein degradation that occurs in sourdoughs, the sourdough proteolysis. In addition, the thesis reports on the protein changes that occur during an emerging cereal fermentation technology, oat bran fermentation.

Sourdough proteolysis plays multiple technological roles in sourdough baking. For instance the degradation of proteins produces amino acids and peptides, which may serve as precursors for flavour-forming compounds. The hydrolysis products may also undergo conversion into antifungal compounds, which can improve the shelf-life of the sourdough bread. In addition, the degradation of gluten proteins in wheat sourdough certainly weakens the wheat dough, whereas the increased solubility of proteins in rye sourdoughs may enhance the structure formation in rye baking. The proteolysis that occurs in sourdoughs thus obviously impacts the overall quality of the sourdough bread. During recent years however, the importance of the cereal proteases in the sourdough proteolysis (compared to that of lactobacilli) has been under scientific discussion. This thesis aims to further this discussion by clarifying the contribution of the cereal proteases to the proteolysis that occurs in sourdoughs.

The literature review part of the thesis summarises the proteolytic systems of cereal grains and examines the technological features that relate to the sourdough proteolysis. In addition, it takes a look at the celiac toxic protein structures and how they can potentially be detoxified by proteolytic enzymes. The experimental part, in turn, studies the protein degradation that happens in traditional wheat and rye sourdoughs and the related protein behaviour that occurs during the acidification process and evaluates some of the factors that affect these. The thesis also introduces the use of a novel sourdough type, a germinated-wheat sourdough that contains a high and diverse proteolytic activity. The aim of studying this highly proteolytic sourdough was to evaluate whether natural food processing and endogenous wheat enzymes could be used to extensively hydrolyse wheat gliadins, the primary triggers in celiac disease, and thus to offer new insights into baking methods for celiac patients. The oat bran

fermentation study, in turn, complements the sourdough studies, since the oat proteins differ drastically from those of the wheat and rye. In addition, the fact that the oat bran fermentation lacks all (cereal-derived) protease activity enables researches to study the effects of the proteolysis that is due to added specific protease without having to contend with interfering naturally occurring enzymes. Such targeted proteolysis may, for instance, be used to produce blood pressure lowering peptides – a theoretical approach for doing this is presented.

2 LITERATURE REVIEW

2.1 The Proteolytic systems of cereal grains

Grains, the seeds of cereal plants, are the ultimate reservoir of their photosynthesis-derived energy, and this reservoir is used for the early development of a new plant. The grains store this energy mainly as starch and storage proteins. The storage proteins primarily serve as a source of amino acids that can be used for protein synthesis (Bewley and Black 1994). But, before this can occur the mobilisation of the storage proteins is necessary, and this requires their proteolytic hydrolysis, or proteolysis. This proteolysis, in turn, requires the presence of proteolytic enzymes (proteases), most of which form during seed germination, although a significant proportion of them are already present in the resting grain. The phenomenon during which the grain takes up water, mobilises its storage molecules, activates enzymes and undergoes innumerable biochemical processes and finally emerges as a seedling is termed germination (Bewley and Black 1994).

One important process that is critical to germination is the conversion of the compact package of storage proteins into peptides and, finally, into soluble amino acids. Prior to that, an arsenal of proteases must be activated/synthesised. These germination-activated proteases, together with those already present in resting seeds, the protein reduction systems of the grains and, of course, the grain storage proteins themselves form an entity that is here referred to as the ‘proteolytic system’ of the grain.

2.1.1 The Prolamins of the *Triticeae* cereals (wheat, rye, barley)

The gluten proteins, the glutenins and gliadins, are the major storage proteins of the wheat grain and make up roughly 75-80% of the total wheat proteins (Osborne 1907). According to the classical Osborne’s classification system (based on the sequential extraction of proteins) the gliadins are the alcohol-soluble proteins of wheat grain whereas the glutenins are the wheat proteins that are soluble in dilute acids (Osborne 1907).

The glutenins are highly polymerised protein molecules that consist of several single glutenin subunits linked together by disulphide bonds. This polymerisation leads to the formation of giant molecules with molecular weights up to several millions (Wahlund et al 1996, Stevenson and Preston 1996). The grouping of glutenin subunits into high molecular weight (HMW) and low molecular weight (LMW) fractions is based on their molecular weights, which are 95-136 kD and 36-44 kD, respectively (Shewry et al 1986). The HMW glutenins are further divided by their molecular weights into the x-types (104-124 kD) and y-types (90-102 kD) and the LMW glutenins are called the B-, C- and D-types, based on their electrophoretic mobilities. The gliadins are monomeric, as they form only intramolecular disulphide bonds, and their grouping into α -, γ - and ω -type gliadins is based on their amino acid compositions; α - and γ -types contain cysteine residues whereas ω -gliadins lack these (Shewry and Tatham 1997). As in wheat, the major storage proteins of rye and barley comprise both monomeric and polymeric proteins. However, unlike the gliadins and glutenins of wheat, the corresponding proteins of rye and barley are each classified under a single term – secalins and hordeins, respectively. Of these, the HMW secalins and D- and B-hordeins are polymeric proteins whereas the ω - and γ -secalins, and γ - and C-hordeins are monomeric (Table 1).

Generally the glutenins, gliadins, secalins and hordeins (along with some other cereal storage proteins) are all included under the term *prolamins* (Shewry et al 1986). The term prolamins refers to the proline (Pro) and glutamine (Gln) rich alcohol-soluble proteins that are typically found in cereals. The rationale for also including the acid-soluble polymeric glutenins, hordeins and secalins under the term ‘prolamins’ rests on the fact that they are soluble in alcohol after they are reduced into monomers.

The prolamins are further divided, based on their molecular weights and sulphur contents, into three subgroups: the HMW prolamins, S-rich prolamins, and S-poor prolamins (Shewry et al 1999) (Table 1). Within the wheat gluten proteins, the HMW prolamins include the HMW glutenins, whereas the LMW glutenins and α - and γ -gliadins belong to the S-rich prolamins subgroup and the S-poor prolamins include the ω -gliadins. With rye and barley the HMW secalins and D-hordeins belong among the HMW prolamins, the γ -secalins and γ -hordeins are the respective S-rich prolamins and the ω -secalins and C-hordeins lack cysteine and thus are included among the S-poor prolamins (Shewry et al 1999). Due to these multiple definitions, it is not surprising that in cereal science the terminology is often used confusedly.

For instance, in celiac disease discussions the term ‘gluten’ refers to any of the prolamins of wheat, rye or barley, whereas in baking circles the term ‘gluten’ is used to refer to the viscoelastic component of the wheat dough structure.

Table 1. The prolamins of the *Triticeae* cereals (wheat, rye, barley). The terminology used depends on the context. For instance, in celiac disease the term ‘gluten’ refers to all of the prolamins of *Triticeae* cereals, whereas in baking technology it usually is a structural term meaning the viscoelastic structure of wheat dough.

Prolamins of the <i>Triticeae</i> cereals			
	Wheat <i>Triticum aestivum</i>	Rye <i>Secale cereale</i>	Barley <i>Hordeum vulgare</i>
HMW prolamins (polymeric)	HMW glutenins	HMW secalins	D-hordeins
S-rich prolamins (polymeric)	LMW glutenins	-	B-hordeins
S-rich prolamins (monomeric)	α - and γ -gliadins	γ -secalins	γ -hordeins
S-poor prolamins (monomeric)	ω -gliadins	ω -secalins	C-hordeins
	Gluten proteins (wheat prolamins)	Secalins (rye prolamins)	Hordeins (barley prolamins)
Gluten (celiac disease)			

2.1.2 The Oat 12 S globulins

The major storage protein group in oat grains (groats) is the globulins, which comprise approximately 75% of the total proteins in the groats (Colyer and Luthe 1984). In the oat endosperm the predominant proteins are the 12 S globulins (Burgess and Miflin 1985). The 12 S globulins are hexameric holoproteins, with M_r of ~320 kD, that are composed of six pairs of acidic and basic polypeptides (Peterson 1978, Walburg and Larkins 1983). The acidic and basic polypeptides are linked together by disulphide bond and they thus form a dimeric globulin subunit with M_r in the range of 50-65 kD (Walburg and Larkins 1983). The variation in the subunit sizes results from the heterogeneity in the sizes of the acidic and basic polypeptides, which vary between 35-40 kD and 20-25 kD, respectively (Walburg and

Larkins 1983). The isoelectric ranges of the acidic and basic polypeptides are between pH 4–5 and pH 7–8, respectively (Walburg and Larkins 1983).

The technological properties of the oat globulins differ from those of other cereal storage proteins. The oat globulins are salt-soluble proteins, which differentiates them from the cereal prolamins. The oat globulins also are very heat-stable proteins and they easily tolerate boiling temperatures, with their thermal denaturation taking place at approximately 110°C (Ma and Harwalkar 1987, Marcone et al 1998). Spectroscopic studies, however, indicate that harsh pH-conditions may change their conformations and elicit an unfolding of their structures (Ma et al 2000, 2001).

2.1.3 Protein reduction systems of wheat grain

Thioredoxin *h* system

The degradation of the storage protein reservoirs in germinating wheat grains begins with the unravelling of the structures of the compact proteins. The compactness of the protein reservoirs is due to the stabilisation of the storage proteins by the disulphide bonding that forms during grain development. Within wheat it is hypothesised that a protein disulphide isomerase (EC 5.3.4.1) manages the folding and assembles the gluten proteins during grain filling by catalysing the formation of disulfide bridges between the gluten proteins by an oxidation process (Bulleid and Freedman 1988, Shimoni et al 1995).

An opposite phenomenon occurs during germination, when the reduction of disulphide bonds takes place. The unravelling of the disulphide linked polymers leads to the loosening of the compact protein structures, which leads to an increased solubility and makes the gluten proteins more susceptible to proteolytic breakdown. One system that has been proposed as being the key actor in the reduction of gluten proteins is the thioredoxin system (Suske et al 1979), which, among the *Triticeae* cereals, has been extensively studied with wheat (see below) and has also recently been characterised in barley (Marx et al 2003).

The thioredoxin system of wheat includes the thioredoxin protein (12 kD), which is activated by a NADPH-dependent thioredoxin reductase (NTR) (Figure 1). The thioredoxins of wheat grain are type-*h* thioredoxins and they efficiently reduce wheat gliadins and LMW glutenins

(Kobrehel et al 1992, Wong et al 1993). The gliadins and glutenins also evidently undergo reduction during germination, and wheat flour apparently contains the required enzyme activities for the activation of the thioredoxin system (Kobrehel et al 1992, Lozano et al 1996). Wong et al (2004a) showed that the wheat thioredoxins may also improve the solubility of wheat gluten proteins.

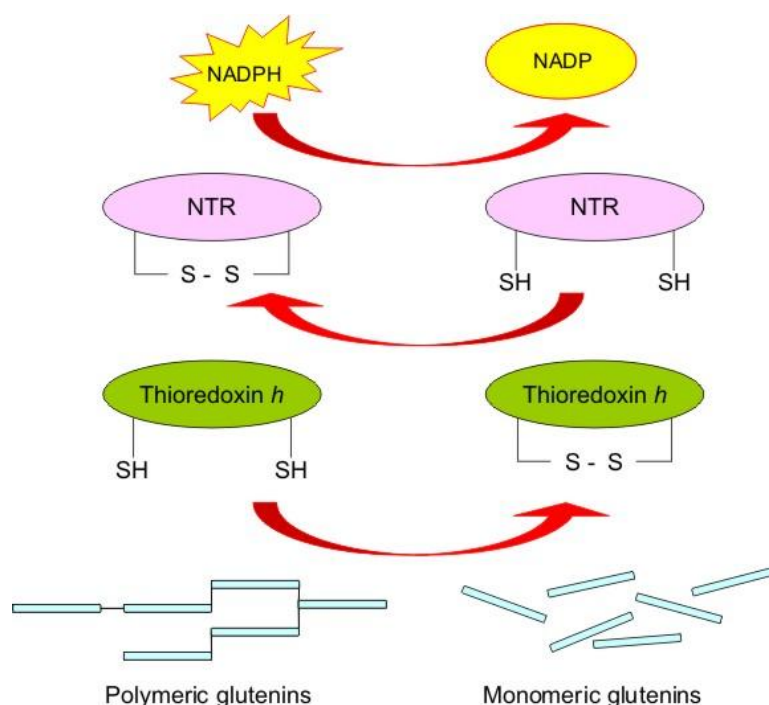


Figure 1. The thioredoxin *h* reduction system specifically reduces disulphide bonds of gluten proteins – with polymeric glutenins this elicits depolymerisation. The system is comprised of a NADPH-dependent thioredoxin reductase (NTR) that activates the thioredoxin *h* protein. The thioredoxins, in turn, reduce the disulphide bonds of glutenins, which causes their depolymerisation. Analogously, the thioredoxin system is suggested to activate and inactivate certain enzymes and enzyme inhibitors, respectively.

The results of Kobrehel et al (1992) and those of Wong et al (1993) indicated that thioredoxins only weakly reduce the HMW glutenins. It seems, however, that in these studies the HMW glutenins were accidentally hydrolysed during the acetic acid extraction by endogenous wheat proteases and thus only few intact HMW glutenins were present to serve as substrates in the experiments. This theory is supported by Jarraud and Kobrehel (2000) who, instead of using an acetic acid extraction, prepared a soap-extract of HMW glutenins; the

results showed that thioredoxins elicited a very efficient reduction of HMW glutenins (Jarraud and Kobrehel 2000).

In addition to its ability to unravel the prolamin structures and thereby increase their solubilities and susceptibilities to proteolysis, the thioredoxin system seems to possess other functionalities that could enhance the macromolecule mobilisation during grain germination. For instance, the thioredoxin system is suggested to be able to activate certain proteolytic enzymes of the cysteine and serine classes (Stephen et al 1993, Besse et al 1996) and to enhance the activity of pullulanase, a starch debranching enzyme (Cho et al 1999), and also to inactivate certain amylase and protease inhibitors (Kobrehel et al 1991, Jiao et al 1992, 1993) – all of these suggested features are likely to promote the macromolecule mobilisation. Recent studies have revealed that the grain thioredoxins may be able to reduce a diverse assortment of potential target proteins (Wong et al 2003, 2004b), which indicates that thioredoxins participate in and regulate numerous unlisted phenomena during the grain development and germination, and some of these may affect the mobilisation of storage proteins. Other potential protein reduction systems besides the thioredoxin system also exist.

Glutathione/Glutathione reductase system

Generally, a glutathione/glutathione reductase (GSH/GR) system is present in practically all organisms and it typically functions as an antioxidant and protects the cells by maintaining their intracellular redox-potential. For instance, oxidative stress induces glutathione reductase (GR) activity, which further increases the pool of active glutathione (GSH), and thus increases the redox-potential. Figure 2 illustrates the GSH/GR system in which the NADPH-dependent GR (EC 1.6.4.2) activates the GSH by reducing the oxidised form of glutathione (GSSG) into two redox-active GSH molecules. Thus, in its active form the glutathione molecule (GSH) is a tripeptide (γ -glutamylcysteinylglycine) while in its oxidised form (GSSG) two glutathione molecules are linked via a disulfide bond.

De Lamotte et al (2000) isolated and characterised a GR from wheat grain. The enzyme appeared to be a typical plant GR, being a dimer that consisted of two 60 kD polypeptides. The pH-optimum of the wheat GR was 7.5-8.0 and 10% of its activity remained at pH 5.5; the GR activity varied but was abundant throughout the grain development (De Lamotte et al 2000).

The presence of GSH in wheat grains was noticed as early as 1936 by Sullivan, and its effects on baking quality have been studied and discussed ever since. It is clear that the addition of

exogenous GSH to wheat doughs gradually weakens their gluten structures (Li et al 2000). However, the role of endogenous GSH, that is present in flours, in baking and its effects have been discussed. This is due to the fact that statistical methods have failed to give correlations for the content of endogenous GSH and the studied baking quality parameters (Bollecker et al 2000, Li et al 2004). In their review, Grosch and Wieser (1999) however, suggested that the well-known improving effects that ascorbic acid has for wheat dough structures probably is based on its ability to oxidise the endogenous GSH molecules to their inactive form (GSSG) (Figure 2). This oxidation of GSH to GSSG would prevent the reduction of disulphides of gluten proteins and thus also the weakening of the dough. This suggestion was verified later by Koehler (2003). The review of Grosch and Wieser (1999) also shows the complexity of redox reactions in wheat doughs, and it is possible that every aspect that is required has not always been taken into account when studying the effects of GSH on baking quality.

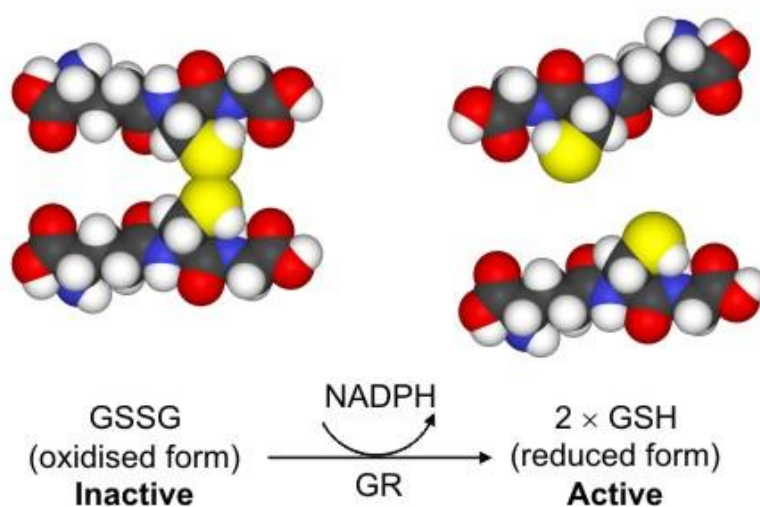


Figure 2. Glutathione/Glutathione reductase system (GSH/GR). A NADPH-dependent glutathione reductase (GR) activates the oxidised form of glutathione (GSSG) by reducing the disulphide bonds that hold two glutathione molecules together, which results in the formation of two active GSH molecules.

Compared to thioredoxins, the ability of GSH to reduce the disulfides of gluten proteins looks weak (Jarraud and Kobrehel 2000). It is, however, obvious that the incorporation of such gluten-active redox compounds into a semi-fluid system (e.g. sourdough with a dough yield of 250) changes the situation radically compared to that of a normal dough. In such slurry-like

suspensions the normal biochemical reactions are likely speeded up, because the enzymes can find their substrates more readily.

It is worth mentioning that the GSH/GR system is often linked to that of the thioredoxin-analogue, glutaredoxin, which could have multiple possible functions in plant cells. The true function of this system remains a mystery in many respects (Rouhier et al 2004). The glutaredoxins have generally been studied with non-plant organisms, and only one cereal (rice) glutaredoxin has been characterised (Minakuchi et al 1994, Sha et al 1997).

A Thiol:protein disulfide oxidoreductase from wheat grain

Recently, Osipova et al (2005) characterised yet another system that may regulate disulphide bonding in wheat grains. The system includes the GSH-dependent enzyme thiol:protein disulfide oxidoreductase (TPDO). The wheat grain TPDO is a dimeric enzyme that consists of two polypeptides with molecular weights of 77 and 73 kD. Incubation of the enzyme with a gluten substrate increased the amount of free thiol groups, indicating that the enzyme reduced some of the disulfide bonds of the gluten. The pH-optimum of the wheat grain TPDO was 7.0, but it remained active down to pH 5.0 (Osipova et al 2005).

2.1.4 The Proteolytic enzymes of resting wheat grains

Nomenclature and definitions of proteolytic enzymes

The nomenclature and definitions of proteolytic enzymes often vary – thus a short description of the practises used in this thesis is necessary. The term proteolytic enzymes refers to all peptide-bond hydrolases (EC 3.4) and the terms proteases or peptidases are often used as synonyms for it. *In this thesis the term proteases is used to refer to all proteolytic enzymes.* The proteases are further grouped into exoproteases and endoproteases on the basis of where the peptide bonds they hydrolyse are located in their substrates. Exoproteases hydrolyse only peptide bonds near the terminal ends of polypeptides or they hydrolyse small peptides. Exoproteases, also often called exopeptidases, are further divided on the basis of their substrate/site specificity. For instance they might hydrolyze peptides at only their amino or carboxy terminii or those that comprise, di-, tri-, or oligopeptides or that contain only prolyl subgroups. *In this thesis the term peptidases always refers to exoproteases.* Whenever necessary, specific prefixed expressions are used (e.g. carboxypeptidase). Endoproteases are

those that cleave peptide bonds that are located in the central part of proteins and their synonyms include the terms proteases, endoproteinases, proteinases, endopeptidases – *in this thesis the term proteinases always refers to endoproteases*. Further classifications of the proteinases are generally based on the chemical structures that are present at their active sites, and the four main classes are the aspartic, cysteine, serine and metalloproteinases. The peptidases may also use the same classification system (e.g. serine carboxypeptidase). In the following sections the proteases that are present in resting wheat grains are reviewed.

Aspartic proteinases and Serine carboxypeptidase II

Although the majority of the cereal proteases appear during the germination of the grain, a pool of aspartic proteinases and serine carboxypeptidases is already present in the resting wheat grain (Table 2) (Belozersky et al 1989, Mikola 1986). These proteases are evenly distributed throughout the grain and are partially associated with gluten. The existence of these gluten-associated proteases means that wheat/gluten processing must be done with care, since their activation can initiate gluten breakdown. Cracker-baking has traditionally exploited this gluten autodigestion (hydrolysis by the endogenous proteases) to weaken the gluten (Pizzinatto and Hoskeney 1980).

Gluten autodigestion. Kawamura and Yonezawa (1982) showed that wheat gluten contained pepstatin-sensitive proteases that decreased the viscosity of gluten suspensions and generated the autodigestion of gluten under acidic conditions. The inhibition by pepstatin indicated that this effect was due to the presence of aspartic proteinases. Bleukx et al (1997) and Capocchi et al (2000) continued the gluten autodigestion studies and verified its occurrence under acidic conditions. During autodigestion at pH 4, the HMW glutenins and LMW glutenins were effectively degraded, while most of the gliadins remained virtually unhydrolysed (Bleukx et al 1997). A new group of proteins of M_r 30-35 kD appeared in their SDS-PAGE gels and these probably were hydrolysis products that arose via gluten breakdown (Bleukx et al 1997). The autodigestion liberated amino acids in a very interesting manner, with leucine, phenylalanine and arginine being the most abundant liberated amino acids (Bleukx et al 1997). The interesting thing about this result is that these amino acids play important roles in the formation of flavour and antifungal compounds in sourdoughs (see sections 2.2.1 and 2.2.3).

Aspartic proteinases. Belozersky et al (1989) isolated an aspartic proteinase with M_r of 58 kD from wheat endosperm tissue. Although the authors stated that the enzyme effectively

hydrolysed gliadins, their results indicate that only weak hydrolysis of gliadins had occurred. In their following study, however, the authors showed that the gliadin hydrolysis was enhanced by the supplementation with a wheat carboxypeptidase (Dunaevsky et al 1989). Galleschi and Felicioli (1994) isolated an aspartic proteinase with M_r of 66.5 kD from wheat bran that hydrolysed wheat bran globulins. The enzyme had a pH-optimum of 3.3 and was activated in the presence of anions that are typically found in aleurone layers (Galleschi and Felicioli 1994). Bleukx et al (1998a) characterised a gluten-associated aspartic proteinase (GIAP) with a dimeric subunit structure (subunits with M_r of 29 and 11 kD) that was structurally similar to the barley aspartic proteinase (EC 3.4.23.40) reported by Runeberg-Roos et al (1991). The pH optimum for GIAP in gluten hydrolysis was 3.0 (Bleukx et al 1998b) and the enzyme was completely inhibited by pepstatin, but it interestingly also lost part of its activity in the presence of a serine protease inhibitor, PMSF (Bleukx et al 1998a). Whether the PMSF-sensitiveness was due to carboxypeptidases is unclear. GIAP hydrolysed glutenins, but had virtually no activity towards gliadins (Bleukx et al 1998b). The specificity of GIAP was typical of that of an aspartic proteinase, as it cleaved peptide bonds adjacent to aromatic and aliphatic amino acids (Bleukx et al 1998b). Bleukx et al (2000) also reported a second gluten-associated aspartic proteinase (GIAP 2) which had a M_r of 67 kD and a pH-optimum of 3.0-3.5. The GIAP 2 was completely inhibited by pepstatin while other inhibitors caused no inhibition. GIAP 2, however, was clearly less active hydrolysing gluten proteins than was GIAP (Bleukx et al 2000).

Serine carboxypeptidase II. In addition to an aspartic proteinase activity, Kawamura and Yonezawa (1982) detected proteases (which had only a minor effect on the gluten viscosity) that hydrolysed a carboxypeptidase substrate at pH 4. The serine protease inhibitor diisopropylfluorophosphate (DFP) arrested this activity indicating serine carboxypeptidases were also associated with gluten (Kawamura and Yonezawa 1982). Capocchi et al (2000) also reported on a PMSF-inhibited carboxypeptidase-like activity that participated in the gluten autodigestion. Dunaevsky et al (1989) reported the isolation of a serine carboxypeptidase from resting wheat grains that preferentially liberated C-terminal aromatic amino acids. The carboxypeptidase had a pH optimum of 4.4 and it was a heterodimer built up of non-covalently linked subunits having M_r of 60 and 63 kD. The enzyme liberated amino acids only weakly from protein substrates, but when the wheat aspartic proteinase (Belozersky et al 1989) was present a synergistic effect was seen (Dunaevsky et al 1989). The enzyme probably was the serine carboxypeptidase II that was earlier reported by Mikola (1986).

In summary, resting wheat grains, which are often used for baking and gluten-starch separations, contain proteolytic activities that are derived mainly from aspartic proteinases and serine carboxypeptidases. Both of these protease groups are active under acidic conditions, are located in the endosperm and are evidently partially associated with gluten proteins. It is likely that these wheat flour proteases work in concert where ever they activate and cause changes in the gluten structures and behaviours.

2.1.5 Proteinases in germinating wheat grains

With barley, the appearance of proteases during germination has been studied extensively, since the barley malt (germinated and kilned barley grains) is used for beer production and protein hydrolysis is one of the critical phenomena necessary for obtaining a successful brewing process. In brewing, the soluble protein hydrolysis products, peptides and amino acids, serve as the main source of nitrogen for yeast growth. This review, however, mainly focuses on the proteases of germinating wheat grain (Table 2) for the following reasons. Firstly, wheat plays a central role in this thesis and the only germinated cereal used in this thesis was wheat. Secondly, a comprehensive review of the barley proteinases has recently been published (Jones 2005). The proteases of germinating oats have also been studied recently (Mikola and Jones 2000a,b, Mikola et al 2001).

In general, the cysteine proteinases are the most important group of proteases for hydrolysing the prolamins in germinating grains. It is, however, obvious that the presence of complementary protease activities intensifies the overall proteolysis and new findings on the role of “underestimated” proteinases may appear. Recently, for instance, it was shown that the metalloproteinases of barley malt are probably important in brewing, as they played a role equal to that of the cysteine proteinases in protein solubilisation during mashing, whereas the serine proteinases were apparently of no technological importance in this system (Jones and Budde 2005).

Cysteine proteinases

Cysteine proteinases are practically absent in developing and mature wheat grains (Domínguez and Cejudo 1996). However, two days after the imbibition of gibberillic acid an accumulation of mRNA encoding cysteine proteinases was induced in aleurone cells (Cejudo

et al 1992) and this was apparently followed by the appearance of the enzymes themselves. Dunaevsky et al (1989) showed that a 35 kD cysteine proteinase effectively hydrolysed wheat gliadins. The efficacy of hydrolysis, however, increased by 4-fold when gliadins were pre-hydrolysed with the aspartic proteinase and carboxypeptidase proteases from resting wheat grain. Domínguez and Cejudo (1995) showed that during wheat germination the proteolytic activity multiplied and that the majority of the approximately 20 proteases were secreted from the aleurone layer and scutellum into the starchy endosperm, while a couple located only in the scutellum. They showed that the proteolytic activity was mainly due to cysteine proteinases (Domínguez and Cejudo 1995). Bottari et al (1996) characterised a cysteine proteinase from germinated durum wheat that had a M_r of 30 kD, that effectively hydrolysed gliadins and whose pH-optimum was 4.3 for the hydrolysis of gliadin. The same proteinase appeared to hydrolyse wheat gluten proteins effectively under reducing conditions at pH 4 (Capocchi et al 2000). Sutoh et al (1999) reported the occurrence of a cysteine proteinase with a M_r of 31 kD in 3-day germinated wheat grains (including in the embryo and seedling). The enzyme had the relatively high pH optimum of 6.0 for hydrolysing a synthetic substrate, but it also hydrolysed wheat gluten proteins, especially the glutenins (Sutoh et al 1999). Taylor and Cuming (1993a) characterised a similar cysteine proteinase that was located in the wheat embryo, that was activated during the first day of germination, and that caused a quick and specific degradation of an albumin-type protein. This cysteine proteinase had a M_r of 38 kD and it operated optimally at the pH-range of 5.5-6.0 (Taylor and Cuming 1993b). Since the enzyme appeared and carried out the protein degradation within the first day after imbibition, it presumably is among the first proteases to be activated in germinating wheat grains.

Metalloproteinases

Despite the substantial metalloproteinase activities that have been shown to occur in both germinated and developing wheat grains (Domínguez and Cejudo 1995 and 1996, Jones and Lookhart 2005) no detailed characterisation of wheat grain metalloproteinases has been carried out. However, the characteristics of some barley metalloproteinases have been studied (Fontanini and Jones 2001). The barley metalloproteinases appeared during the first day of germination and hydrolysed gelatin optimally at pH 8.0 (Fontanini and Jones 2001). Regardless of their neutral pH-optima they apparently hydrolysed D-hordeins even at pH 4, but the rate of D-hordein degradation was greater at pH 8 (Fontanini and Jones 2001). The barley metalloproteinases were located mainly in the aleurone layer, but some activity was also present in the scutellum (Fontanini and Jones 2001).

Serine proteinases

Thiocalsin is a 14 kD serine proteinase that was found in 2-day germinated wheat grains (Besse et al 1996). After the enzyme was treated with active thioredoxin and, in the presence of 20 μM Ca^{2+} , it efficiently hydrolysed wheat glutenins and gliadins. Interestingly, thiocalsin was practically unable to hydrolyse other protein substrates that were tested, including edestin, gelatin, casein and haemoglobin. The optimum pH for thiocalsin was pH 5 and the enzyme was only partly inhibited with PMSF, whereas another serine protease inhibitor (AEBSF) efficiently arrested its activity. Another wheat serine proteinase was isolated from embryos and seedlings of 3-day germinated wheat (Sutoh et al 1999). It hydrolysed gluten proteins, showing a preference for gliadins at pH 7.5, and was inhibited by DFP.

Aspartic proteinases

The aspartic proteinases of wheat grain were dealt above in chapter 2.1.4, so they are not dealt with here. Their activities apparently remain relatively stable throughout germination, as their activities in the resting and in 5-day germinated barley grains was virtually the same (Wrobel and Jones 1992).

2.1.6. Peptidases involved in wheat germination

Carboxypeptidases

Serine carboxypeptidases are abundant in both resting and germinating wheat grains and they operate under acidic pH conditions (Preston and Kruger 1976, Mikola 1986). Mikola (1986) detected five acid carboxypeptidases in the endosperms of germinating wheat grains and termed these as wheat carboxypeptidases I to V, like the corresponding barley carboxypeptidases (Mikola 1983). They hydrolysed dipeptides optimally under mildly acidic conditions (pH 4.4-5.8). Of the five carboxypeptidases, wheat carboxypeptidase I (EC 3.4.16.5) and wheat carboxypeptidase II (EC 3.4.16.6) were the major carboxypeptidases of wheat grain. The wheat carboxypeptidase III is nowadays included under the same EC-number (EC 3.4.16.5) as carboxypeptidase I (Table 2). The wheat carboxypeptidases IV and V are actually prolyl carboxypeptidases and are therefore discussed under the next subtitle.

Wheat carboxypeptidase II was abundant in the resting grains but as germination proceeded its activity decreased. It had a M_r of 120 kD and its ability to cleave the dipeptide Z-Ala-Arg distinguished it from the other four carboxypeptidases (Mikola 1986). The wheat carboxypeptidase I effectively hydrolysed Z-Phe-Ala and had a M_r of 120 kD. It was absent from resting grains but activated during germination and reached its maximum activity after 3-days of germination (Mikola 1986). Wheat carboxypeptidase III preferentially hydrolysed the dipeptidyl substrate Z-Ala-Phe and reached its activity maximum after 3-days of germination. Compared to other four enzymes, the wheat carboxypeptidase III was distinguished by its low (40 kD) M_r (Mikola 1986).

Table 2. The proteolytic enzymes of resting and germinating wheat grains. *Italic lettering is used for barley-derived information that is expected to also be relevant for wheat.*

	Protease EC-number	Location	pH-range /optima	References
Resting	Aspartic proteinases <i>EC 3.4.23.40</i>	Endosperm, bran, gluten-associated	3 3-3.5	Belozersky et al 1989, Bleukx et al 1998b, 2000
	Carboxypeptidase II EC 3.4.16.6	Endosperm, bran, gluten-associated	4.5-6	Mikola 1986
Germinating	Cysteine proteinase	Aleurone	4	Dunaevsky et al 1989, Bottari et al 1996
	Cysteine proteinase	Embryo	5.5-6	Taylor and Cumming 1993ab, Sutoh et al 1999,
	Serine proteinase	Endosperm Embryo	5 7.5	Besse et al 1996 Sutoh et al 1999
	Metalloproteinases	<i>Aleurone,</i> <i>scutellum</i>	8	Domínquez & Cejudo 1995, <i>Fontanini and Jones 2001</i>
	Carboxypeptidases IV and V	Endosperm	4.5-6	Mikola 1986
	Carboxypeptidases I and III EC 3.4.16.5	Aleurone, Scutellum, Endosperm	4.5-6	Mikola 1986
	<i>DPP IV</i> <i>EC 3.4.14.5</i>	<i>Embryo</i>	7	<i>Davy et al 2000</i>
	Aminopeptidases	Embryo/Seedling	7.5	Waters and Dalling 1979
	Prolyl aminopeptidase EC 3.4.11.5	Seedling	7.5	Waters and Dalling 1983
	Oligopeptidase B EC 3.4.21.83	Embryo	8.5	Tsuji et al 2004

Proline specific peptidases

This group of enzymes is of special interest to the beer industry, since these enzymes help to hydrolyse the proline-rich proteins and peptides of malt – this may prevent the formation of haze in beer (Siebert 1999). This is one reason why brewers are particularly interested in the proline-specific enzymes (Simpson 2001) and why the enzyme industry has tried to answer the brewer's needs by offering such enzymes (Lopez and Edens 2005). The proline specific proteases may also play a role in the treatment of celiac disease, as is reviewed in section 2.3.2.

Generally, the presence of a proline residue in an amino acid chain causes its conformation to be inflexible, and this renders it resistant to hydrolysis by most proteases. The proteolytic cleavage of proline-containing peptides thus requires specific proteases. Since the major cereal storage proteins, the prolamins, contain large amounts of proline, it is obvious that the cereal plant itself must possess tools for degrading proteins containing proline. In fact, Mikola and Mikola (1980) showed that free proline accumulated in the starchy endosperms of germinating barley grains. This also occurred when isolated starchy endosperms were incubated at pH 5, and DFP totally prevented the accumulation. This finding indicates that the proteases present in the barley endosperm are capable of extensively hydrolysing the hordeins.

Wheat grain contains proline specific carboxypeptidases; the carboxypeptidases IV and V are both found in the endosperms of wheat grain (Mikola 1986). Their activities require peptide substrates that have a proline residue in the penultimate position of their C-terminal ends. Low levels of carboxypeptidase IV and V activities are already present in the resting wheat grains and these activities increase during germination. At pH 4.4, their pH optima, the wheat carboxypeptidases IV (M_r 165 kD) and V (M_r 150 kD) showed identical specificities towards several proline-containing substrates (Mikola 1986).

Davy et al (2000) purified a barley malt dipeptidyl peptidase IV (DPP IV, EC 3.4.14.5) that had a M_r of 105 kD, a pH optimum of 7.2, and that presumably located in the malt embryo. This DPP IV is a serine class peptidase that preferentially liberates dipeptides from the N-terminal side of polypeptides (Xaa-Xbb-↓-Xcc). Preferably this occurs when proline locates in the Xbb-position, as long as the Xcc is not also a proline residue. The authors concluded that DPP IV probably operates in the embryo of the malted barley grain (Davy et al 2000).

A prolyl aminopeptidase (EC 3.4.11.5) from the primary leaf (shoot) of germinated wheat has also been characterised (Waters and Dalling 1983). It hydrolysed only dipeptides that had N-terminal proline residues (Pro-Leu, Pro-Gly, Pro-Tyr). Inhibitor tests showed that the enzyme belonged to the cysteine-class. Its pH-optimum was 7.4 and it had a M_r of ~400 kD (Waters and Dalling 1983).

Other wheat peptidases

Tsuji et al (2004) identified a serine oligopeptidase (oligopeptidase B, EC 3.4.21.83) from wheat embryos that hydrolysed peptide bonds on the carboxyl side of basic amino acids. The oligopeptidase had a M_r of 81 kD and a pH-optimum of 8.5 (Tsuji et al 2004). Waters and Dalling (1979) showed that two aminopeptidase activities, both having pH optima of 7.4, occurred in wheat grains and were especially prevalent in the growing tissues.

2.1.7 Summary

Gluten proteins, the polymeric glutenins and monomeric gliadins, are the storage proteins of wheat grain and their solubilities increase under acidic conditions. During the grain-filling stage of wheat maturation, the gluten proteins are compactly packed into the grain to serve as an initial nitrogen source when the grain takes up water and germinates. In their compact forms (with intra- and inter-molecular disulphide bonds) the gluten proteins are unavailable for mobilisation, so an unravelling of their compact protein structures is necessary before they can be utilized. Protein disulfide bond reduction systems, such as the thioredoxin *h* system of wheat, can efficiently unravel the compact structures of these reservoir proteins. The thioredoxin system may also enhance the protein mobilisation by activating certain hydrolytic enzymes and possibly by inactivating some proteinase inhibitors. The actual enzymatic protein hydrolysis, termed proteolysis, is carried out by the proteases of the wheat grain. The resting wheat grain contains aspartic proteinases and carboxypeptidase II (both of which are active under acidic conditions) that presumably initiate the proteolysis in the endosperm. As the germination proceeds, an arsenal of proteases appears, of which the cysteine-class proteinases are apparently the most important. These proteases operate over diverse pH-ranges and in general it seems that the closer a particular enzyme locates to the embryo, the higher its pH-optimum is (Table 2).

2.2 The Technological impacts of proteolysis in sourdoughs

Sourdough fermentation is a time-consuming pre-baking process and to be worthwhile, it must therefore add substantial value for the final product, the sourdough bread. Some of the technological improvements that can be obtained by conducting sourdough baking are improved product flavour and shelf-life, and also enhancements in the characteristics of the final dough and bread are often listed as benefits (e.g. Hammes and Gänzle 1998). In addition, the use of sourdoughs in baking reduces the need of additives and probably increases the positive image of the products – these beneficiary characteristics encourage bakeries to use sourdoughs. However, with wheat sourdoughs especially, the improvements in the bread and dough properties may be difficult to achieve. This is because the acidic flavour is not always perceived as being palatable in wheat bread, whereas the extensive gluten breakdown that occurs during the sourdough process may interfere with developing good doughs. These concerns determine the requirements for optimising the process and selecting starters for sourdough baking. Of the baking-technological characteristics that can be attributed to sourdough fermentations, at least the flavour, dough structure and product shelf-life are related to the protein degradation that occurs during the sourdough fermentation, the sourdough proteolysis.

2.2.1 The Formation of flavour compounds from amino acids

Mechanisms of flavour compound formation

A comprehensive review on sourdough baking and its effect on the formation of flavour compounds has recently been published by two leading researchers in the field (Hansen and Schieberle 2005). The overview presented here focuses on the formation of amino acid-derived flavour compounds during sourdough baking (Table 3).

During sourdough fermentation, cereal proteins are degraded into free amino acids that may be converted to flavour compounds; i.e. the liberated amino acids act as flavour precursors.

The conversion of these precursors to actual flavour compounds may occur during baking via thermal conversions or during the sourdough fermentation via microbial conversions. Both the sourdough yeasts and LAB may facilitate the flavour formation, either directly via metabolising amino acids to flavor compounds or indirectly by transforming them into secondary compounds that can serve as new precursors for further conversions.

Examples of some direct microbial conversions are the generation of 3-methylbutanal and 3-methylbutanol from leucine or of 2-phenylethanol from phenylalanine via the Ehrlich-pathway of *Saccharomyces cerevisiae* (Ehrlich 1907). Generally *S. cerevisiae* uses the Ehrlich-pathway for the degradation of aromatic (Phe, Tyr, Trp) and branched chain amino acids (Ile, Leu, Val). In fact, the ability of *S. cerevisiae* to produce flavour compounds relates to its inability to use all amino acids as a carbon source. This leads it to take up only ammonium ions, which in turn causes the release of flavour-active aldehydes and isoalcohols (Sentheshanmuganathan 1960).

The indirect flavour compound formation (i.e. generation of new precursors) occurs, for example, in the conversion of arginine into ornithine by *Lactobacillus pontis* (Vogel et al 1994) and *Lactobacillus sanfranciscensis* (De Angelis et al 2002). Ornithine is a precursor for the roasty-flavoured 2-acetyl-1-pyrroline (Table 3).

Amino acids may also undergo conversion to flavour compounds during baking via thermal conversions such as the Strecker degradation of amino acids. The Strecker degradation of leucine produces its corresponding aldehydes and acids, namely 3-methylbutanal and 3-methylbutanoic acid and similar reactions with phenylalanine generate phenylacetaldehyde and phenylacetic acid (Hofmann et al 2000) (Table 3).

Typical amino acid-derived flavour compounds in sourdough bread

Wheat sourdough bread. Hansen and Hansen (1996) related the improvements in the flavour of wheat sourdough bread to the increased contents of several amino acid-derived flavour compounds: 2/3-methylbutanol, 2-methylpropanoic acid, 3-methylbutanoic acid and 2-phenylethanol. Czerny and Schieberle (2002) showed practically identical results using wheat sourdoughs. In these sourdoughs increases in the concentrations of 2-methylbutanal, 3-methylbutanal, and 2- and 3-methylbutanoic acid were evident. According to Schieberle and Grosch (1994) the ornithine derivative 2-acetyl-1-pyrroline facilitates the formation of the typical crust flavour in wheat sourdough bread.

Rye sourdough bread. Hansen et al (1989) studied the sensory properties of rye sourdough bread crumbs and emphasized the significance of both 3-methylbutanal and 2-phenylethanol for the flavour. Kirchhoff and Schieberle (2001) detected twelve compounds that affected the flavour of rye sourdough bread crumbs and of these, five (3-methylbutanal, phenylacetaldehyde, methional, 2- and 3-methylbutanoic acid) were derivatives of amino acids. Methional also appeared to be the key flavour compound in the crust of rye sourdough bread (Schieberle and Grosch 1994). Kirchhoff and Schieberle (2002) also pointed out that of the five compounds the concentrations of 3-methylbutanal and of the 2- and 3-methylbutanoic acid actually increased during rye sourdough fermentations. Very similar findings regarding the increase of 3-methylbutanal were seen with extruded sourdoughs (Heiniö et al 2003).

In summary, proteolysis produces amino acids that can act as flavour precursors. The conversion of these into flavour compounds may occur via the microbial conversion during sourdough fermentation step or via thermal conversions during baking. Overall, sourdough yeasts appear to be more important than LAB in forming flavours. For instance *S. cerevisiae* produces numerous flavour compounds via the Ehrlich-degradation of amino acids, whereas the LAB may possess strain-specific features such as the ability to convert arginine to ornithine (Table 3). The thermal conversion of amino acids can occur via Strecker-reactions and this produces typical flavour compounds in sourdough breads (Table 3). The evidence indicates that leucine, isoleucine, phenylalanine and methionine are among the most important amino acids for the flavour formation that occurs in sourdough baking.

To date, the research relating to the chemistry of *sourdoughs and flavours* has focused on the volatile flavour compounds. In their review, Hansen and Schieberle (2005) actually highlighted the necessity for future studies to clarify the roles that non-volatile flavour compounds play in the flavour of sourdoughs. It is rather surprising that no studies on how small peptides affect the flavour characteristics of sourdough breads have been carried out, since it is well known that many of them produce bitter tastes.

Table 3. Amino acids that can act as flavour precursors in sourdoughs. A selection of amino acids that probably play important roles in the formation of flavour compounds, and their occurrences in wheat and rye prolamins. The pathways whereby the amino acids can be converted to flavour compounds and the odour of the flavour compounds are also listed.

Amino acid	Amount ¹ Mol-% of the prolamins		Conversion pathway	Flavour compound or <i>Secondary precursor</i>	Odour
	Wheat	Rye			
Leu	7.1	5.7	Ehrlich ² /Strecker ³	3-methylbutanal	malty ⁵
			Ehrlich	3-methylbutanol	malty ⁶
			Strecker	3-methylbutanoic acid	sweaty ⁵
Ile	3.7	3.0	Ehrlich/Strecker	2-methylbutanal	malty ⁵
			Ehrlich	2-methylbutanol	
			Strecker	2-methylbutanoic acid	sweaty ⁵
Phe	4.5	4.6	Ehrlich	2-phenylethanol	flowery ⁶
			Ehrlich/Strecker	phenyl acetaldehyde	honey-like ⁵
			Strecker	phenylacetic acid	sweet, honey-like ⁶
Arg	2.1	1.5	ADI ⁴	Ornithine (Orn)	-
Orn	-	-		2-acetyl-1-pyrroline	roasty ⁵
Val	3.8	4.4	Ehrlich/Strecker	2-methylpropanal	malty ⁶
			Strecker	2-methylpropanoic acid	
Met	1.2	1.1	Strecker	methional	cooked potato ⁵

¹ Data collected from Shewry and Miflin 1985

² Ehrlich 1907

³ see Hofmann et al 2000

⁴ see Christensen et al 1999

⁵ Hansen and Schieberle 2004

⁶ Kirchhoff and Schieberle 2001

2.2.2 Dough rheology

Gluten proteins and rheology

Generally, the formation of wheat doughs with good viscoelastic properties is completely dependent on the gluten proteins, the glutenins and gliadins, which form the gluten network during the preparation of doughs. The polymeric HMW glutenins play an especially important role in determining the overall baking quality of wheat, as they provide the strength and elasticity to wheat doughs. The viscous properties of wheat doughs are associated with the gliadins and LMW glutenins. It is therefore evident that if the polymeric structures of the HMW glutenins are unravelled, the formation of the gluten network will be interfered with and the resulting dough will be weaker and less elastic. This is a well-recognised and exploited phenomenon in cracker baking, but similar reactions likely occur in sourdoughs as well.

Rheological changes in cracker baking

For baking crackers, weak or weakened wheat flours are used to produce a dough (sponge) that has a high extensibility and low resistance to extension. Such a stretchy sponge is obtained after a yeast fermentation of up to 24 hr in the presence of salt and soda additives. Pizzinatto and Hosney (1980) have described the effects of fermentation on the properties of cracker sponges. In summary, their extensograph work with cracker sponge (without additives) showed that prolonged fermentation times led to a decrease in the sponge strength. They concluded that this weakening was likely due to the decrease in pH from approximately pH 5 to 4 that directly affected the gluten proteins and, in addition, activated the flour enzymes (Pizzinatto and Hosney 1980). Doescher and Hosney (1985) reported that other reactions besides proteolytic breakdown seemed to contribute to the rheological changes that occurred during the initial stages of sponge fermentations and that antioxidants seemed to prevent these reactions. It is possible that the phenomenon responsible for the observed weakening of the sponges was the depolymerisation of the gluten proteins.

Rheological changes and sourdough baking

Studies dealing with the rheology of wheat sourdough baking often conclude that an unravelling of the gluten network is the causal factor for the altered rheologies. It is, however,

necessary to emphasize that the rheological behaviours of natural sourdoughs greatly differ from those of chemically acidified wheat doughs. Generally, a natural sourdough results in a less elastic dough than its chemically acidified counterpart (Clarke et al 2002).

Clarke et al (2004) showed that the use of a wheat sourdough in dough preparation decreased the elasticity of the final dough when it was compared to a dough prepared without sourdough addition. In addition, the laser micrographs verified that there was a breakdown in the gluten network (Clarke et al 2004). Similar findings were observed with wheat doughs whose elasticity and firmness decreased whenever fermented sourdough was used in their preparation (Clarke et al 2002, Angioloni et al 2006). Evidently, there is no direct relationship between the acidity of a dough and its change in elastic behaviour, as was shown in a series of studies that used a relatively simplified approach. Wehrle et al (1997) added lactic acid to a dough and observed the increases in its elasticity and firmness. Clarke et al (2002) found that lactic acid had no effect on the elastic behaviour of wheat doughs. However, the results of Schober et al (2003) showed that the swelling of gluten pieces in lactic acid buffer increased their elasticity.

Contrary to the wheat gluten proteins, the rye secalins are unable to develop gluten-like structures with viscoelastic behaviour, and when added to wheat doughs the secalins may, in fact, disrupt the formation of the gluten (Kipp et al 1996). Soluble rye proteins and protein hydrolysis products may, however, play a role in the formation of continuous structures in rye doughs. This hypothesis is based on the study of Meuser et al (2001) which showed that soluble rye proteins (during mixing) are able to form foams. Such protein foams, together with the solubilised arabinoxylans, might be important factors in the structure formation during rye sourdough baking, and the sourdough fermentation could enhance the foam formation by increasing the amount of soluble rye proteins.

In summary, sourdough fermentations decrease the elastic properties of wheat doughs and the protein degradation that occurs weakens the wheat dough. These phenomena determine how much fermented sourdough can be used in wheat dough preparation. In rye sourdoughs, however, the acidification and proteolysis increase the amounts of soluble rye proteins and peptides, which may enhance the dough properties by favouring the formation of foam structures. Sourdough proteolysis may enhance this foam formation by generating increased amounts of soluble rye proteins and peptides.

2.2.3 Formation of antifungal compounds

One interesting area of sourdough research that relates to sourdough proteolysis is the study of the formation of antifungal compounds. These compounds may be generated during the metabolism of amino acids by LAB and they may be important for extending the shelf-lives of sourdough breads.

Phenyllactic acid (PLA) is apparently the most potent amino acid-derived antifungal compound that (until today) is found in sourdoughs. Recently, Lavermicocca et al (2000) showed that a *Lactobacillus plantarum* strain produced antifungal activity when grown in a wheat flour autolysate, and that this activity prevented the growth of *Aspergillus niger* in bread. The antifungal activity was mainly due to the presence of PLA and, to a lesser degree, to 4-hydroxy-phenyllactic acid (OH-PLA) (Lavermicocca et al 2000). In a follow-up study, PLA and OH-PLA were shown to be metabolites of phenylalanine and tyrosine, respectively (Valerio et al 2004). The PLA clearly delayed the growth of fungi, especially that of strains of *Aspergillus flavus* and *Penicillium roqueforti* (Lavermicocca et al 2003). The inhibitory activity of PLA was seen at pH 4, and the presence of lactic and acetic acids evidently strengthened the inhibition (Lavermicocca 2003). The formation of PLA was increased by the addition of dipeptides to the growth media of *Lactobacillus sanfranciscensis* and *Lactobacillus plantarum* (Vermeulen et al 2006); this increase in PLA formation was based on the preference of LAB to uptake peptides rather than free amino acids. However, the addition of peptides to sourdoughs failed to enhance the formation of PLA presumably because of the high levels of protein hydrolysate products (peptides) that were already present in the sourdough (Vermeulen et al 2006).

2.3 Celiac disease and gluten peptides

Gluten proteins, particularly the gliadins, are the causal factor in celiac disease (CD). In CD the ingestion of gluten-containing foods leads to an inflammation of the small intestine. The inflammation is a consequence of a series of reactions (for a review, see Koning 2005). First, the human leukocyte antigens HLA-DQ2 and HLA-DQ8 react with the epitope peptides that are present in gluten proteins. This antigenic reaction leads to the activation of T-cells and to their consequentially expressing inflammatory cytokines. This inflammation then activates the cross-linking enzyme called tissue transglutaminase (tTGase), which increases the affinity of the gluten peptides for the HLA-DQ2 and HLA-DQ8 molecules by deamidating their glutamine residue(s). This deamidation increases the affinity of the gluten peptides, which accelerates the series of reactions, which eventually results in the atrophying of the intestine. Currently the only treatment for CD is a life-long gluten free diet which, in practice, means that foods containing wheat, rye, and barley proteins must be avoided. The prevalent current opinion is that proline-rich peptide sequences that are present in gliadins and other prolamins are the causal epitope in CD.

2.3.1 Toxic gluten peptides in celiac disease

Different approaches have been used to solve the mystery behind the recognised linkage between gluten proteins and CD. Prior to today's practise of exploiting the T-cell reactivity in the research, limited-scale studies were carried out with selected protein fractions or hydrolysates and their corresponding effects on the mucosal physiology were evaluated (for a review, see Wieser 1995). Sturgess et al (1994) were among the first to clearly show that a single gliadin peptide could elicit toxicity *in vivo*. They discovered that the ingestion of an α -gliadin 19-mer polypeptide, consisting of 19 amino acids (Table 4), caused atrophy of the villi of celiac patients. A year later, Marsh et al (1995) showed that a 13-residue (Table 4) peptide of the same protein, but lacking the last six amino acids of the 19-mer, was toxic as well. Kasarda (1997) searched databases to explore for potential celiac-toxic peptides and, based on its presence in wheat, barley, and rye (but not in oats), highlighted the possible importance of the hexapeptide QQQPFP in causing CD.

After Molberg et al (1997) had documented the common occurrence of DQ2-restricted T-cells in celiac patients' biopsies a new approach was introduced. The philosophy behind this approach is to study the T-cell responses that are elicited by various gliadin peptides. Sjöström et al (1998) were the first to describe a gliadin peptide, 10-mer (Table 4), that elicited the proliferation of CD-associated T-cells. After this study, numerous publications based on T-cell responses have appeared and the T-cell proliferation assay is currently the predominant indicative assay that is used in CD-toxicity research. The numerous T-cell response studies have clearly indicated that T-cell activation takes place in response to a variety of gluten peptides (Anderson et al 2000, Arentz-Hansen et al 2000a,b, 2002, Vader et al 2002, Spaenij-Dekking et al 2004) including glutenin peptides (Vader et al 2002, Molberg et al 2003, Dewar et al 2006), and that the responses are strongly patient-dependent (Siegel et al 2006). Today, the tendency is also to explore for homologs of previously identified toxic peptides in databases, to synthesize these homologs and, in turn, to test their abilities to elicit T-cell responses (Vader et al 2002, 2003).

Table 4. A selection of celiac-toxic peptide sequences. The common feature shared by these toxic peptides is that they have Pro-Gln-rich amino acid sequences. Database homology searches can easily multiply the number of such potential peptides that could be listed.

Peptide	Sequence	Origin and location ¹	Reference
33-mer	LQLQFPFPQQLPYPQQLPYPQQLPYPQPQPF	α -2 gliadin, 56-88	Shan et al 2002
26-mer	FLQPQQFPQPQQPYPQQPQQPFPQ	γ -5 gliadin, 26-51	Shan et al 2005
20-mer	LQPQQFPQPQQPYPQQPQ	γ -5 gliadin, 60-79	Arentz-Hansen et al 2002
20-mer	QQQQPPFSQQQSPFSQQQQ	glutenin	Vader et al 2002
19-mer	LGQQQFPFPQQPYPQPQPF	A-gliadin, 31-49	Sturgess et al 1994
17-mer	QLQFPQPQLPYPQPQS	A-gliadin, 57-73	Anderson et al 2000
15-mer	VQGQGIIQPQQPAQL	γ -gliadin	Vader et al 2002
15-mer	QQPPFSQQQQQLPQ	glutenin	Vader et al 2002
14-mer	PQPQLPYPQPQLPY	α -2 gliadin, 62-75	Arentz-Hansen et al 2000
13-mer	LGQQQFPFPQQPY	A-gliadin, 31-43	Marsh et al 1995
12-mer	FSQPQQQFPQPQ	γ -5 gliadin, 102-113	Arentz-Hansen et al 2002
12-mer	QLQFPQPQLPY	α -9 gliadin, 57-68	Arentz-Hansen et al 2000
10-mer	QPQQSFPQQQ	γ -gliadin	Sjöström et al 1998

¹ α 2-gliadin of Arentz-Hansen 2000; γ 5-gliadin of Arentz-Hansen 2002; A-gliadin of Kasarda et al 1984

Another approach for resolving the CD mystery utilizes the hypothesis that the celiac toxicity of a peptide strongly correlates with its proteolytic resistance (Hausch et al 2002, Shan et al 2002, 2005). It has long been known that ‘the toxic factor’ is resistant to digestion by pepsin and trypsin (Frazer 1959). More recently, Hausch et al (2002) noticed that certain proline- and glutamine-rich polypeptides were resistant to hydrolysis by pancreatic enzymes and were only weakly degraded by brush-border membrane (BBM) peptidases. The hydrolysis of these immunodominant peptides by BBM peptidases accumulated hydrolysis products that still contained typical T-cell recognised sequences (Hausch et al 2002). In a hydrolysate prepared by sequentially reacting α -gliadin with pepsin and a pancreatic protease mixture, Shan et al (2002) discovered an intact polypeptide, a 33-mer (Table 4). The 33-mer was rich in proline (13 residues) and glutamine (10) and thus possessed a typical proteolytically resistant structure. The authors showed that the 33-mer reacted with tTGase and noticed that it contained three nonapeptides that had previously been shown to cause T-cell proliferation; both findings (tTGase and T-cell responses) indicated that the 33-mer should probably be toxic (Shan et al 2002). A database search found that the prolamins of *Triticeae* cereals contained several amino acid sequences that were homologous to those of the 33-mer (Shan et al 2002). In a follow-up study, Shan et al (2005) showed that the α -gliadin lost its T-cell proliferation effect when the amino acids comprising the 33-mer were omitted from its structure. This indicated that the 33-mer was the only fragment in the used recombinant α -gliadin that was able to elicit toxicity. They also discovered a 26-mer (Table 4) proteolytically-resistant fragment from γ -gliadin that was similar to the 33-mer (Shan et al 2005). The 26-mer also contained known T-cell epitopes in its sequence and reacted with tTGase, indicating it behaved like a celiac toxic peptide (Shan et al 2005). A database survey revealed many prolamins-derived candidate peptides (at least 60) that could potentially behave like the 26-mer and 33-mer (Shan et al 2005). It is thus evident that no single prolamins fragment is the causal factor in CD, but that the toxic peptides share a common feature – their Pro-Gln-rich peptide sequence.

It is noteworthy that most of today’s studies are being made with recombinant gliadins such as α -2 gliadin (Arentz-Hansen et al 2000b), from which Shan et al (2002) isolated the 33-mer. Natural wheat gliadins evidently contain similar structures, but it still needs to be borne in mind that this research is mainly operating with model systems.

2.3.2 The Detoxification of gluten peptides by proteolysis

Sollid and Khosla (2005) have listed various approaches that could be used for treating CD. Of these, using proteolytic enzymes to detoxify harmful gluten peptides falls within the scope of this thesis. The principle behind this approach is to proteolytically hydrolyse the toxic gluten epitopes into non-toxic hydrolysis products. Generally, two alternative hydrolysis philosophies exist: to hydrolyse them after the gluten has been ingested, in the gastrointestinal (GI) tract (the medical approach) or to hydrolyse them prior to the gluten having been ingested, during food processing (the food technological approach). The detoxification of gluten by proteolysis is not a novel idea and neither is the use of more than one protease in an effective detoxification procedure. For instance, Messer et al (1964) showed that crude papain (which contains several diverse proteolytic activities) could detoxify gluten, whereas one purified papain proteinase failed to detoxify it. The following sections deal with more recent studies relating to the 'proteolytic gluten detoxification'.

POP, prolyl oligopeptidase from *Flavobacterium meningosepticum*

The need of additional protease activities that could hydrolyse gluten proteins was verified by Hausch et al (2002) who showed that the typical T-cell epitopes were resistant to the sequential hydrolyses that were carried out by the GI and brush border membrane (BBM) enzymes. On the other hand, an effective hydrolysis did take place when the BBM enzymes were supplemented with a bacterial prolyl oligopeptidase (POP). In their 33-mer work, Shan et al (2002) showed that in the presence of POP the 33-mer was efficiently broke down into compounds that no longer elicited the proliferation of T-cells. In addition, the 26-mer that was very resistant to GI and BBM enzymes was rapidly hydrolysed after POP supplementation (Piper et al 2004). *In vivo* studies with rats supported these findings, as the perfusion of POP together with gluten peptides into the rat intestine accelerated the digestion of the gluten peptide *in vivo* by 50-100% (Piper et al 2004). The particular POP that was used in these studies originated from *Flavobacterium meningosepticum* (Chevallier et al 1992), and it showed higher hydrolytic activity against the 33-mer than did two other POP enzymes (Shan et al 2004).

Some contradictory results were noticed with the use of POP. Shan et al (2004) reported that the POP was rather stable under acidic conditions, whereas Stepniak et al (2006) reported the opposite – that the POP lost half of its activity when incubated for only 15 min at pH 2.0. The sensitiveness of the POP to hydrolysis by the gastric enzyme pepsin is also not clear. Shan et

al (2004) and Stepniak et al (2006) reported the POP being sensitive to pepsin hydrolysis whereas another study showed that it was stable to pepsin (Matysiak-Budnik et al 2005). Matysiak-Budnik et al (2005), however, were suspicious about the efficacy of the POP-therapy for treating CD, since a high concentration of the enzyme was required to completely detoxify the 33-mer. Likewise, Stepniak et al (2006) questioned the practicality of using the POP enzymes due to the fact that they are only active at neutral pH values, they are inactivated by pepsin and acidic conditions, they prefer to degrade small substrates and they are unable to degrade intact gluten molecules.

PEP, prolyl endoprotease from *Aspergillus niger*

Stepniak et al (2006) therefore introduced the use of a new enzyme – a prolyl endoprotease (PEP) from *Aspergillus niger* that (to some extent) lacked the shortcomings of POP. The PEP retained 20% of its activity and was stable under gastric conditions (pH 2.0), even though it operated optimally at pH 4.5 and retained 20% of its activity at pH 8.0. PEP, working alone, detoxified a gluten-derived substrate preparation and, when used as part of hydrolysis system that mimicked the GI hydrolysis milieu, the used enzyme cocktail efficiently hydrolysed intact gluten in the presence of PEP. This, in turn, led to the detoxification of T-cell epitopes present in the gluten (Stepniak et al 2006). The authors evaluated the general efficacy of PEP for hydrolysing gluten peptides and concluded that PEP hydrolysed the gluten peptides 60 times faster than POP did. It is noteworthy that PEP is the same enzyme that has reported been found useful also for beer clarification and for debittering protein hydrolysates (Lopez and Edens 2005, Edens et al 2005).

EP-B2, a cysteine proteinase from *Hordeum vulgare* (barley)

A barley cysteine proteinase called EP-B is responsible for hydrolysing the bulk of the hordeins during barley germination (Zhang and Jones 1996). Recently, a recombinant barley cysteine proteinase proenzyme (proEP-B2) was expressed in *Escherichia coli* and the activity of the mature enzyme (EP-B2) was profiled among the proenzyme conversion (Bethune et al 2006). Interestingly, the EP-B2 was resistant to pepsin hydrolysis, but was degraded by trypsin, which predicts that it could potentially work only in the gastric phase of digestion. Overall, the proEP-B2 activation (its conversion into EP-B2) took place under acidic conditions optimally only below pH 4. The activity of the EP-B2 for hydrolysing a synthetic substrate (Z-Phe-Arg-pNA), however, surprisingly increased with increasing pH and the activity was optimal at the highest pH tested, pH 7 (Bethune et al 2006). However, the pH-optimum for

the hydrolysis of the synthetic substrate by the recombinant enzyme preparation was pH 4.5, which is the pH-optimum that is typically reported for barley cysteine proteinases (for a review, see Jones 2005). Thus, despite the mature EP-B2 showed highest activity at pH 7 the conversion of proEP-B2 required more acidic conditions and the compromise pH for the conversion and hydrolysis was 4.5 (Bethune et al 2006).

Nevertheless, when the proEP-B2 was incubated with α -gliadin at pH 4.5, the substrate protein was effectively hydrolysed. Gliadin hydrolysis also occurred at pH 3.0, but a comparison of the number of cleavage sites revealed that a more intensive hydrolysis had occurred at pH 4.5. The previously studied 33-mer polypeptide was also degraded by the EP-B2 (Bethune et al 2006). These results indicate that a single cysteine proteinase, EP-B2, is able to hydrolyse and thereby detoxify gliadin peptides.

Glutenase, a combination of EP-B2 and POP

As would be expected, when gluten was sequentially hydrolyzed with a cocktail of EP-B2 accompanied by gastric pepsin under acidic conditions, and subsequently by a secondary hydrolysis with POP mixed with pancreatic enzymes (trypsin, chymotrypsin, elastase and carboxypeptidase A) at neutral conditions, it was highly degraded and very effectively detoxified (Siegel et al 2006). The enzyme cocktail that contained EP-B2 and POP was called 'glutenase'.

In summary, the detoxification of gluten peptides has evidently become a potential therapeutic method for treating celiac patients. Most of the reported studies seem to prefer the use of an enhanced GI-tract digestion, rather than carrying out the hydrolysis during food processing. In this thesis, however, a food technology approach that is based on using raw materials with highly active and diverse proteolytic enzymes (germinated wheat) and a pH-dynamic food processing method (sourdough fermentation), is presented.

2.4 Objectives of this study

- To describe and explain the prolamin degradation that occurs in sourdoughs
- To clarify the role of cereal proteases in the sourdough proteolysis events
- To evaluate the extent of prolamin hydrolysis in a wheat sourdough made with wheat grains of widely diverse proteolytic activity
- To examine the phenomena that occur in the oat 12 S globulins during the fermentation of oat bran

3 MATERIALS AND METHODS

3.1 Sourdough and Oat bran fermentations

Four different cereal fermentations were used to conduct the research that is reported in Studies **I-IV**:

- I** WSD, wheat sourdough
- II** RSD, rye sourdough
- III** GWSD, germinated-wheat sourdough
- IV** OBF, oat bran fermentation

Cereal raw materials used in the fermentations (Table 5) included commercial wheat flour (WF) and whole grain wheat flour (GF) (**I**), ground meals of whole grain rye of two cultivars (RM-Am and RM-Ak) (**II**), ground meal of germinated wheat grains (GW) (see below) (**III**), and the oat bran concentrate (OBC) (**IV**). More precise information on the cereal raw materials that were used appears in the Table 5 and in the original articles.

The GW was prepared as follows. Wheat grains (*Triticum aestivum* L, cv. Kruunu) were germinated in 6×800 g batches with a commercial pilot malting equipment (Joe White Malting Systems, Melbourne, Australia) in darkness at VTT (the Technical Research Centre of Finland). The germination process was selected according to the results of preliminary trials and the details were as followed. The grains were steeped at 15°C for 48 hr (16 hr wet steep, 8 hr air rest, 8 hr wet, 15 hr air, 1 hr wet). This resulted in grains with moisture content of ~47% (w/w). The steeped grains were germinated at 15°C for 3, 5 or 8 days. Each sample was divided into two proportions, of which one underwent Pilsner-drying (PD) and the other one freeze-drying (FD). In PD the temperature was sequentially raised (by using continuous warmed air-flow) from 50°C to 85°C during the 14 hr drying process. Rootlets were removed from dry samples. The grains were ground with a cyclone sample mill (Tecator Cyclotec 1093, Höganäs, Sweden) equipped with a 0.5 mm screen and the protease activities of the ground samples were determined. Finally, a mixture of 8-day germinated wheat grains (equal volume-proportions of PD and FD grains) were used to prepare the GW that was used as a raw material for GWSD fermentation (**III**).

Fermentation starters varied among the individual sourdoughs. The fermentations were initiated by the addition of a seed rye sourdough (**I, II**), a combination of pure cultures (**I**), or a single pure culture strain (**III, IV**) (Table 5). The rye sourdough seed was a laboratory rye sourdough that contained a mixed population of homo- and heterofermentative lactic acid bacteria and a yeast strain of *Candida milleri*. The pure cultures included *Lactobacillus brevis* VTT E 95612 (**I**), *Lactobacillus plantarum* VTT E 78076 (**I**), *Saccharomyces cerevisiae* VTT B 81047 (**I**), *Lactobacillus brevis* (Florapan L62, Lallemand SA, Blagnac Cedex, France) (**III**), and *Lactobacillus rhamnosus* (HOLDBAC™ LC, Danisco A/S, Niebüll, Germany) (**IV**). All pure cultures, except that of *L. rhamnosus* used in the OBF experiments, were precultured prior to the inoculations in cereal-water suspensions.

Fermentation conditions and dough yield (DY) varied as well. In preparing the sourdoughs, the inoculated cereal-water mixtures were incubated in water-baths at 30°C (**I, II**) or 34°C (**III**) for 16 hr (**I**) or 24 hr (**II, III**), whereas the OBF was held at 37°C for 24 hr (**IV**). The DY of the sourdoughs varied in the range of 230-260, which practically means that the sourdoughs were slurries. The theoretical DY in OBF was approximately 2000. The DY value is calculated as follows: $DY = 100 \times \frac{\text{dough weight}}{\text{flour weight}}$

Control incubations were used to evaluate the roles of different protease sources in the sourdough proteolysis. In the antibiotic buffered incubations (ABI) the wheat flours or rye meals were suspended in appropriate buffers that contained an antibiotic mixture (Thiele et al 2002) and incubated at a constant pH for 16-24 hr (**I, II**). A control wheat sourdough (CSD) and an inactive wheat sourdough-like ferment (ISD) were used as reference materials when studying the protein degradation in the GWSD experiment (**III**). The CSD was a wheat sourdough prepared with ungerminated whole grain-derived wheat meal, whereas the ISD was prepared by boiling the ground wheat meals in the presence of a heat-stable alpha-amylase; the amylase treatment allowed the comparison of samples containing similar meal-water ratios by preventing the gelatinisation of the starch.

Fermentation parameters were determined to ensure that successful fermentations were obtained. The progress of the *in situ* fermentations was monitored by continuously recording their pH-values (**I-IV**), whereas the total titratable acidity (TTA) values of samples gave more relevant information about the formation of organic acids during the fermentations (**II-IV**).

The concentrations of lactobacilli were occasionally determined by cultivating diluted samples on MRS (de Man, Rogosa, Sharpe) agar plates.

3.2 Protein analyses

Sequential protein extractions, based on Osborne's wheat protein classification system (Osborne 1907), differed among the individual studies (**I-IV**). Generally, either a buffered or a non-buffered extraction procedure produced three or four protein solubility fractions (Table 5). The first step generally extracted the readily soluble proteins and contained either water-soluble albumins or the salt-soluble proteins (albumins and globulins). The second extraction step, utilizing aqueous alcohol as a solvent, extracted the weakly polymeric and monomeric prolamins and the final extraction step, using a reducing SDS-PAGE sample buffer (SDS-SB) dissolved the polymeric or residual prolamins/proteins. In Study **I**, an extraction step that utilized non-reductive SDS-SB (prior to the reductive SDS-SB extraction) dissolved the moderately polymeric prolamins.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) was the main analytical tool used for monitoring the protein changes that occurred during the fermentations. The protein fractions were mixed with the reductive SDS-SB and heated for 2-3 min at 100°C. The protein migration rates were generally analysed with 12% Tris-HCl gels (Bio-Rad Laboratories, Hercules, CA) (**I, II**) or 12% Tris-Hepes-SDS gels (Pierce Biotechnology, Rockford, IL) (**III, IV**). Some analyses of the wheat ABI samples were carried out with 4-12% Bis-Tris gels (Criterion XT, Bio-Rad) (**I**). A typical SDS-PAGE analysis with the 12% Tris-HCl gels used MiniProtean II electrophoresis system (Bio-Rad). The SDS-PAGE samples (10 µL) were loaded in sample-wells of the gels. The current was set to be 10 mA/gel. After the samples appeared as a uniform front-lane, the current was raised to 20 mA/gel. The electrophoresis took approximately 90 to 100 min. The gels were removed and stained separately in plastic containers overnight using 70 mL of a Coomassie solution. The Coomassie solution contained 10 mL of a Coomassie stock-solution, 30 mL 12% TCA and 30 mL of distilled water; the Coomassie stock-solution was an ethanol solution that contained 1 g of Serva Blue R (Serva Electrophoresis GmbH, Heidelberg, Germany) per litre of 96% (v/v) ethanol. The overnight-stained gels were destained by incubating the gels in distilled water for at least 24 hr and after that the gels were photographed on a light-table.

The Determination of the free amino nitrogen (FAN) levels of water-soluble fractions (**II**, **III**) followed a standard procedure (ASBC 1992). Briefly, 1 vol of the ninhydrin reagent and 2 vol of diluted sample were mixed and heated at 100°C for 16 min. After being allowed to cool at 20°C for 20 min, the reaction mixture was diluted with 5 vol of the KIO₃ solution and the absorbance of the solution was read at 570 nm against a zero time-sample. The FAN content was calculated by comparison with the absorbance readings obtained with a glycine standard.

The Soluble-protein contents of enzyme (**III**) and protein (**IV**) extracts were occasionally determined by using either methods based on Lowry et al (1951) or Bradford (1976). In both cases assay kits of Bio-Rad were used (RC DC Protein assay or Bio-Rad protein assay, respectively).

An ELISA method (Valdés et al 2003) was used to evaluate the extent of gliadin hydrolysis by determining the prolamin contents of a straight alcohol-soluble extract of GWSD (**III**) by the use of a commercial kit and following the manufacturer's instructions (Transia Plate *Prolamins*, Raisio Diagnostics, Finland).

3.3 The Profiling of proteolytic activities

Protease extracts were prepared according to Zhang and Jones (1995) (**I**, **II**) or Jones et al (2000) (**III**) with some modifications. In a typical extraction procedure, ground grains or flours and sodium acetate buffer (50-100 mM, pH 5) were incubated at +5°C for 30-60 min with continuous shaking and then centrifuged at the same temperature. The supernatant obtained was the crude protease extract, which was used in hydrolyses as such (**I**, **II**) or concentrated with ammonium sulphate precipitation prior used in hydrolyses (**I**) or filtered and dialysed against dilute buffer prior further testing (**III**).

The Hydrolysis of azosubstrates resulted in the liberation of dye into the reaction medium, and the extent of proteolysis was determined by measuring the absorbance of the sample at 440 nm. The hydrolysis of azocasein (Megazyme International, Wicklow, Ireland) determined the general proteolytic activity of the proteinase extracts (**I**, **II**) and it was the protein substrate used in the inhibitor studies (**I-III**). Azocasein is hydrolysed by all four of the proteinase

groups which is an advantage in comparison to using azogelatin, which is not hydrolysed by the aspartic proteinases. At the same time, using azocasein is sometimes a disadvantage because it precipitates under acidic pH conditions (Jones et al 1998). In this study, azocasein was hydrolysed at pH values that ranged from, 4.3-4.9. After 1-3 hr of incubation at 40°C the reactions were terminated by adding trichloroacetic acid (TCA) and, after cooling and centrifugation, the 440 nm absorbance of the supernatant was measured. Azogelatin is soluble over a wide pH range (Jones et al 1998) and in this study it was used to measure the general proteinase activities at different pH-values (**III**). The procedure used in Study **III** followed that originally used by Jones et al (1998), with minor modifications.

Inhibitor studies were used to determine the proteolytic profiles of sourdough and flour/meal extracts (**I-III**). The inhibitors used and their concentrations in the azocasein reactions were adapted from the work of Zhang and Jones (1995). Each inhibitor was incubated for ~5 min with the protease extract before the actual reaction was initiated by adding the azocasein substrate solution. The degree of the inhibitory effect was expressed either as the remaining relative activity (%) or as relative inhibition (%).

Edestin hydrolysis in gels made it possible to visualise the differences among the overall proteolytic activities that were present in sourdoughs and cereal extracts (**I**). Edestin (a hemp seed storage globulin) was incorporated into a native electrophoresis gel as described by Wrobel and Jones (1992). The principle of this method is first to drive the proteins (enzymes) present in the protease extract into the gel under cold conditions to avoid enzyme inactivation and denaturation. After this electrophoresis, the gels are incubated in appropriate incubation buffers and after incubation the protein gels are stained. If the substrate protein is hydrolysed by some of the enzymes present in the electrophoregram, no staining occurs at the location of an active enzyme, since the protein that was originally there has been hydrolyzed. A comparison of the intensities of unstained areas allows the making of a qualitative comparison between the proteolytic activities of the different enzymes. Generally, information about the various enzymes is gained by varying the incubation conditions (pH, temperature and time), but the addition of inhibitors or activators to the incubations can also allow additional information about the enzymes to be obtained.

Determination of the activities of the proline specific enzymes POP and DPP IV used specific substrates that were designed for measuring these enzymes. The prolyl oligopeptidase (POP) activity was determined using a modified method (Venäläinen et al 2002) that utilized

the substrate Suc-Gly-Pro-AMC. Similarly, the determination of dipeptidyl peptidase IV (DPP IV) activity used H-Gly-Pro-AMC as the substrate. In both cases the liberation of the fluorescent AMC group allowed the specific activity to be determined (**III**).

Table 5. A summary on the experimental procedures that were used in studies **I-IV**.

S	Research subject	Raw material	Starters ^a	T, t	DY	Control	E	Protein fractions	Protein analysis	Proteolytic activity
I	WSD	WF GF	RS <i>L. brevis</i> <i>L. plantarum</i> <i>S. cerevisiae</i>	30°C, 16 hr	230/250	ABI	B	- albumin-globulin ^b - weakly polymeric - moderately polymeric - highly polymeric	- SDS-PAGE - FAN ^c	- azocasein - edestin - inhibitor studies
II	RSD	RM-Am RM-Ak	RS	30°C, 24 hr	265	ABI	NB	- water-soluble - alcohol-soluble - residual proteins	- SDS-PAGE - FAN	- azocasein - inhibitor studies
III	GWSD	GW	<i>L. brevis</i>	34°C, 24 hr	260	CSD ISD	B	- water-soluble - alcohol-soluble - SDS-SB soluble	- SDS-PAGE - FAN - ELISA	- azogelatin - POP and DPP IV - inhibitor studies
IV	OBF	OBC	<i>L. rhamnosus</i>	37°C, 24 hr	~2000	-	B NB	- salt-soluble - alcohol-soluble ^b - SDS-SB soluble	- SDS-PAGE - Lowry ^c	-

S, study number; WSD, wheat sourdough; RSD, rye sourdough; GWSD, germinated-wheat sourdough; OBF, oat bran fermentation; WF, wheat flour; GF, whole grain wheat flour; R-Am, ground meal of rye (*Secale cereale* L. cv. Amilo); R-Ak, ground meal of rye (*Secale cereale* L. cv. Akusti); GW, ground meal of germinated wheat (*Triticum aestivum* L. cv. Kruunu); OBC, oat bran concentrate; RS, seed rye sourdough; T, incubation temperature; t, incubation time; DY, dough yield; ABI, antibiotic buffered incubation; CSD, control sourdough; ISD, inactive wheat sourdough-like ferment; B, buffered extraction procedure; NB, non-buffered extraction procedure; SDS-SB, SDS-PAGE sample buffer, SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; FAN, free amino nitrogen; ELISA, enzyme linked immunosorbent assay; POP, prolyl oligopeptidase; DPP IV, dipeptidyl peptidase IV

^a more precise information is listed in the text and original articles

^b fraction was analysed but found irrelevant (data thus not presented)

^c figures presented in the thesis but that are not shown in the original articles

4 RESULTS AND DISCUSSION

4.1 The Degradation of prolamins in sourdoughs (I-III)

Sourdough fermentations offer nearly ideal conditions for the degradation of cereal prolamins, since the sourdough is a pH-dynamic semi-fluid system that allows the activation of cereal proteases that have varying pH optima. In addition, the acidity of the fermentation increases the solubility of the prolamins, which makes them more susceptible for proteolytic breakdown.

This research utilized three types of sourdoughs, of which two are traditionally used in wheat and rye baking. The third sourdough-type was a germinated-wheat sourdough that was prepared from germinated wheat grains that contained many diverse, high level proteolytic activities. This novel sourdough type was used to evaluate the capacity of endogenous cereal proteases to extensively hydrolyse the wheat prolamins during sourdough fermentation. The ultimate goal was to obtain new insights that can be used when baking for celiac disease patients. The following sections present the sourdough studies case by case and discuss the key protein degradation phenomena that were observed and their impacts on baking.

4.1.1 HMW glutenin degradation in wheat sourdoughs (I)

The degradation of polymeric HMW glutenins was evident during the wheat sourdough fermentations (Figure 3). The degradation apparently comprised two overlapping but distinguishable stages: the unravelling of the highly polymeric HMW glutenin structure and the actual hydrolysis of the HMW glutenins. These phenomena are designated as the depolymerisation and the proteolysis, respectively (Figure 3).

Depolymerisation

The term depolymerisation refers to the unravelling of the highly polymeric HMW glutenins into weakly polymeric HMW glutenins. The depolymerisation of HMW glutenins was apparent after 4 hr of fermentation, as the solubility of the HMW glutenins decreased in

reductive SDS-SB (Figure 3A). This indicates that the degree of polymerisation of the HMW glutenins had decreased. At the same time, the solubility of the HMW glutenins in 55% 1-propanol increased (Figure 3B), suggesting that increased amounts of weakly polymeric and monomeric HMW glutenins were present. Thiele et al (2004) observed a very similar apparent depolymerisation of HMW glutenins in wheat sourdoughs by using other chromatographic methods (SEC, RP-HPLC) and different solvents.

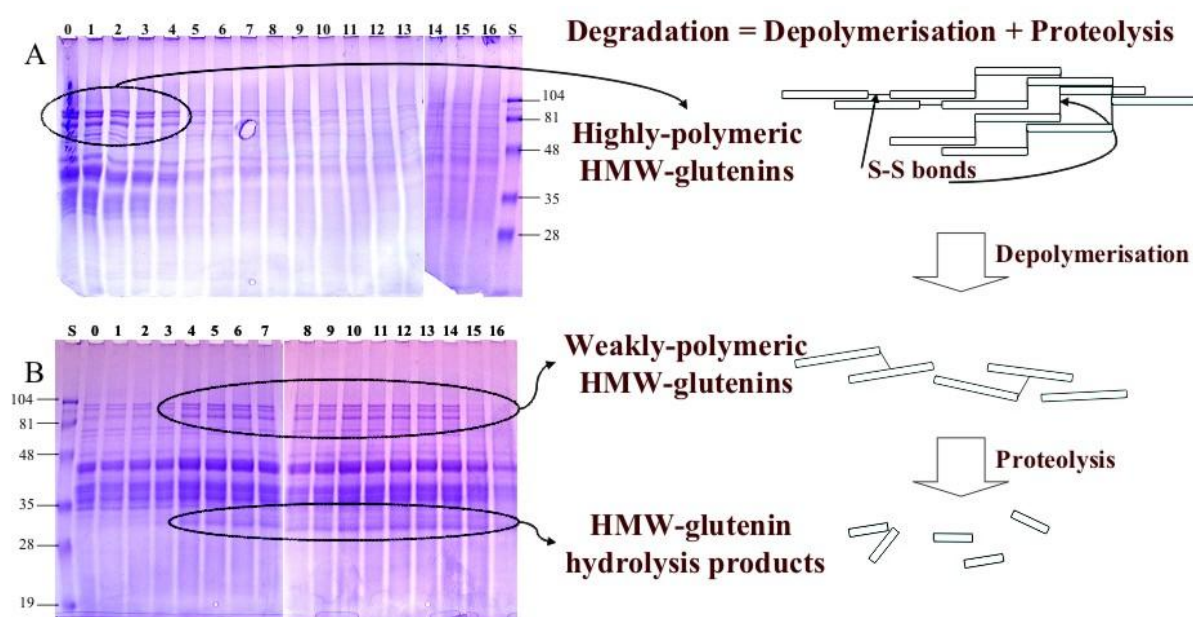


Figure 3. Illustration on the HMW glutenin degradation that occurs during WSD fermentation. On the left, SDS-PAGE gels A and B contain the highly polymeric and the weakly polymeric gluten proteins, respectively. The fermentation time is shown above the gels. On the right is a schematic representation of the reduction-induced depolymerisation and subsequent proteolysis of HMW glutenins.

At least two possible mechanisms for the depolymerisation exist: a reduction-induced depolymerisation and a proteolysis-induced depolymerisation. The reduction-induced depolymerisation would require the presence of reducing compounds that could reduce the disulphide bonds between the HMW glutenin subunits ($S-S \rightarrow HS-SH$). Such reductive compounds could originate from the sourdough microbes and/or from the cereal ingredients that were used. Recently, Vermeulen et al (2006) showed that a sourdough strain of *Lactobacillus sanfranciscensis* expressed glutathione reductase-like activity that, during sourdough fermentations, activated exogenous glutathione molecules ($GSSG \rightarrow 2 \times GSH$). In

addition, when the same LAB strain was used as a starter and the thiol concentrations of the sourdough were compared with those of a chemically acidified dough, it appeared that the number of free thiol groups increased in the sequentially extracted propanol fraction of the fermented sourdough (Vermeulen et al 2006). This finding is perfectly compatible with the present observations on the HMW glutenin shifting from the highly polymeric fraction (reductive SDS-SB extracts) into weakly polymeric fractions (propanol extracts) that is observable in Figure 3.

These findings strongly indicate that a reduction-induced depolymerisation likely occurs in wheat sourdough, which practically always contains at least one strain of heterofermentative lactobacillus. In addition to the heterofermentative lactobacilli the sourdough yeasts presumably also contain glutathione and thioredoxin systems (Grant 2001) that may facilitate the redox-dependent reactions in sourdoughs. But as the complexity of the sourdough system is well recognised, it is probable that analogous compounds that are also present in cereals play a role in the depolymerisation. In fact, it is possible that the reductases of sourdough microbes may be involved in activating the endogenous protein reduction systems of cereals.

Wheat grain itself contains effective reduction systems for depolymerising gluten (as reviewed in the section 2.1.3). The wheat thioredoxin system (Figure 1) in particular reduces gluten proteins *in vitro* (Jarraud and Kobrehel 2000) and increases their solubilities by reducing disulphide bonds (Wong et al 2004a). Thioredoxin-induced depolymerisation also occurs during the germination process (Kobrehel et al 1992). The thioredoxin system thus evidently works during germination, but whether it is also activated in sourdoughs is unclear. The activation of such a system in the sourdough environment, however, is reasonable, since thioredoxin and the NADPH-dependent thioredoxin reductase (NTR) are abundant in mature wheat grains, and thus also in wheat meals (Lozano et al 1996). Kobrehel et al (1992) showed that wheat meal also contains the necessary catalysts for maintaining adequate NADPH-levels, but at the same time they concluded that the NTR might be the overall limiting factor for the entire wheat meal thioredoxin system. If so, it is obvious that the presence of sourdough microbes could enhance the activity of the protein reduction systems of cereals by providing, for instance, NADPH or reductase enzymes.

An alternative explanation for the depolymerisation is that proteolysis-induced depolymerisation occurred. This also would reduce the polymerisation of the HMW glutenins and thus elicit the solubility shifts that were detectable on the SDS-PAGE gels. In simplified

terms, a proteolytic cleavage of a polymeric HMW glutenin molecule would produce two less-polymeric HMW glutenin molecules that would presumably have improved solubilities in aqueous alcohol. The DTT that is present in the SDS-PAGE sample preparation buffer ensures that the HMW glutenin subunits and their hydrolysis products will be detectable on SDS-PAGE gels (Figure 3). This proteolysis theory would also explain the simultaneous appearance of hydrolysis products in the propanol extract (Figure 3B, 4 hr sample). This proteolysis-induced depolymerisation hypothesis is supported by Thiele et al (2004) who showed that the depolymerisation was dependent on acidic conditions and did not take place under neutral conditions. If the depolymerisation really is dependent on an acidic pH this would support the proteolysis-induced depolymerisation theory, since the wheat proteases are most active under acidic conditions, whereas the protein reductases preferably work under more neutral conditions.

Proteolysis in wheat sourdoughs

The proteolytic degradation of HMW glutenins occurred during wheat sourdough fermentations. This was clearly observable on SDS-PAGE gels as the HMW glutenins disappeared at the end of the 16 hr fermentation (Figure 3B). Similar degradation also took place in the wheat sourdoughs that were started with pure cultures and that were made with both white wheat flour and whole grain flour. The degradation was faster in the latter, which was due to the higher proteolytic activity of the whole grain flour (Table 6). The proteolysis generated alcohol-soluble hydrolysis products with M_r of ~30 kD that were apparently resistant to further hydrolysis and that accumulated in the propanol extracts (Figure 3B). Bleukx et al (1997) reported the formation of similar hydrolysis products during gluten autodigestions under acidic pH conditions. The proteolysis also produced soluble hydrolysis products that were detectable as increased levels of free amino nitrogen (Figure 4).

A similar HMW glutenin degradation was also observable in the antibiotic buffered incubation at pH 3.7, which proves that the proteases of wheat flour can hydrolyse HMW glutenins under acidic conditions in the absence of starter microbes. No hydrolysis occurred at pH 5.3. The results from the edestin hydrolysis experiments verified that both cereal and sourdough extracts showed proteolytic activity towards edestin at pH 3.5, but not at pH 5.5.

Table 6. The proteolytic activity of some rye and wheat raw materials with the substrate azocasein. Hydrolysis was at pH 4.3 (data adapted from Study I).

Raw material	Proteolytic activity (AU)
Rye flour (whole grain)	1.67 ± 0.02
Whole grain wheat flour	1.35 ± 0.01
Wheat flour	0.43 ± 0.02
Rye bran	2.70 ± 0.05
Wheat bran	1.51 ± 0.01

^bAU (arbitrary unit) = the increase in A 440 nm per minute $\times 1000 \pm$ S.D.

Inhibitor studies with sourdough-derived enzyme extracts confirmed that aspartic proteinases (inhibited by pepstatin A) were the dominant proteases in sourdoughs, although some PMSF-inhibited serine-class protease activity was also present. This protease profile fits well with the one that is typically present in resting wheat grains (see section 2.1.4). It thus appears that the serine protease activity that was detected in sourdough extracts originated from wheat carboxypeptidase II, rather than from serine proteinases from LAB. This is reasonable, since the serine carboxypeptidases are active under mildly acidic conditions (Preston and Kruger 1976, Mikola 1986) whereas the serine proteinases of LAB possess more neutral pH-optima (Kunji et al 1996).

Our findings of the dominant role that aspartic proteinases play in sourdough proteolysis are supported by several other studies. Vermeulen et al (2005) successfully diminished the formation of protein hydrolysis products by supplementing wheat sourdoughs with an aspartic proteinase inhibitor whereas Thiele et al (2002, 2003, 2004) definitely concluded that the flour proteases that are active under acidic conditions were responsible for the gluten degradation in wheat sourdoughs. In addition, the work of Kawamura and Yonezawa (1982) unequivocally showed that gluten-associated aspartic proteinases and serine carboxypeptidases hydrolysed gluten proteins under acidic conditions. Other studies have also proved that this pair of proteases hydrolyses glutenins under acidic conditions (Dunaevsky et al 1989, Bleukx et al 1997, Cappochi et al 2000). Rheological studies with cracker sponges and sourdoughs have also consistently shown that cereal proteases are responsible for the

changes in protein structures that lead to their altered dough rheologies (Pizzinatto and Hoseney 1980, Schober et al 2003, Clarke et al 2004).

The findings from this study thus apparently agree with most studies that have dealt with the sourdough proteolysis, even though there is still a partial disagreement with the studies that assume that the proteolytic activity of LAB is crucial for sourdough proteolysis (Gobbetti et al 1996, Di Cagno et al 2002, 2004, Rizzello et al 2006). Gobbetti et al (1996) measured the proteolytic enzyme activities of several sourdough lactobacilli and detected some gluten degradation. However, the gluten that was used as substrate was prepared from wheat flour and presumably contained some of the gluten-associated wheat proteases. In addition, Gobbetti et al (1996) showed that probable gluten-derived peptides were released during sourdough fermentations and they connected this release to the proteolytic activity of the LAB. It is noteworthy that these sourdoughs probably had very stiff consistencies, as their DYs were ~150. In addition, the control sourdoughs were chemically acidified with lactic acid, which presumably made the doughs even stiffer (see Clarke et al 2002). In general, it can be presumed that the low mobility of water and solubles retards biochemical reactions. In this thesis, the sourdoughs were slurries that had DY values of 230-260. Thiele et al (2002, 2003, 2004) also prepared slurry-like sourdoughs that had DY values between 200-400.

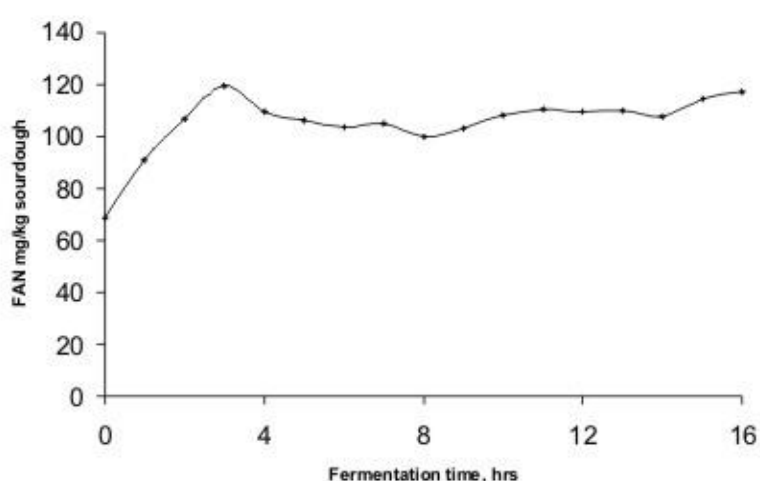


Figure 4. The development of free amino nitrogen (FAN) during the WSD fermentation.

The controversial role played by the LAB proteases does not, however, indicate that the LAB biochemistry is totally meaningless relative to sourdough proteolysis. On the contrary, during their growth in sourdoughs LAB produce organic acids, which gradually turn the sourdough from a neutral system into an acidic one. The acidic conditions, in turn, activate the cereal proteases and also improve the solubility of gluten proteins and thereby render them more susceptible to proteolytic breakdown. The LAB utilize proteolysis-derived peptides for their growth and presumably excrete any unnecessary amino acids back to the sourdough medium. These excreted amino acids (if not taken up by yeast) appear in the FAN determinations and also serve as potential precursors for flavour and antifungal compounds. In this respect the LAB possess strain-dependent specificities such as the ability to convert arginine to ornithine (a precursor for the acetyl-1-pyrroline, see 2.2.1) (Vogel et al 1994) or the conversion of phenylalanine to phenyllactic acid (an antifungal compound, see 2.2.3) (Lavermicocca et al 2000). In addition, the glutathione-reductase activity of the heterofermentative LAB may enhance the protein degradation that occurs in sourdoughs (Vermeulen et al 2006). Thus, the LAB, rather than playing meaningless roles in the sourdough proteolysis, actually play multiple roles. They create favourable conditions for the sourdough proteolysis and also participate in converting some of the proteolysis products into technologically important compounds.

Technological aspect: Repolymerisation of HMW glutenins

The sourdough proteolysis produces hydrolysis products in the sourdough that may serve as precursors for flavour or antifungal compounds as reviewed above (see 2.2). In addition, the degradation of HMW glutenins certainly affects rheological properties of the dough. Considering how important the gluten network formation in wheat doughs is, knowing the mechanism of how depolymerisation occurs is very important. If the depolymerisation of the HMW glutenins is induced by the reduction of disulphide bonds, a repolymerisation of the HMW glutenin subunits (prior to their extensive proteolytic breakdown) could reconstitute the polymer structures in the dough preparation. For instance, the addition of hexose oxidases (HOX) may indirectly promote such repolymerisation, because HOX produces H_2O_2 that is capable of oxidising gluten thiol groups into disulphide bonds (Poulsen and Bak Høstrup 1998). If, however, the depolymerisation is proteolytically induced, such a reconstitution of disulphide bonds with HOX is not possible, but other cross-linking enzymes could be tested in order to see whether they can improve the dough structure. Usually, however, the dosage of

fermented sourdough that is added to the wheat dough preparation is adjusted to optimise the structure of the final dough.

To summarise, in wheat sourdoughs the degradation of HMW glutenins can be the product of two overlapping phenomena: depolymerisation and proteolysis (Figure 3). The depolymerisation decreases the size of the highly polymeric glutenin molecules until they become weakly polymeric molecules that dissolve in aqueous alcohol. Whether the depolymerisation is induced by the reduction of disulphide bonds between the glutenin subunits or by proteolysis-induced depolymerisation is unclear. The results of Thiele et al (2004) indicate that the depolymerisation is dependent on acidic conditions, which favours the proteolysis-induced depolymerisation hypothesis, since the flour proteases are active under such conditions. Alternatively, Vermeulen et al (2006) have highlighted the meaningfulness of heterofermentative lactobacilli and their reductive compounds in the depolymerisation, which would support the reduction-induced depolymerisation hypothesis. Overall, however, it is reasonable to conclude that the protein reduction systems probably play a substantial role in the biochemical events that occur in sourdoughs.

The actual proteolytic degradation of HMW glutenins is mainly carried out by the cereal aspartic proteinases. The LAB-derived proteases seem to play a minor role or no role in the sourdough proteolysis. This is reasonable, since the cell-wall anchored serine proteinases of LAB have a neutral pH-optima and their effectiveness in sourdoughs thus is questionable. Instead of a direct proteolytic contribution, the LAB create an ideal environment for the cereal proteases to operate, since the proteases present in resting grains and thus in flours typically are active under the acidic conditions that are created in sourdoughs by LAB. In addition, the solubility of gluten proteins increases during sourdough fermentations due to the depolymerisation and acidification, which renders them more susceptible to proteolytic degradation.

4.1.2 Secalin hydrolysis in rye sourdoughs (II)

Proteolysis occurred quickly in rye sourdoughs, and the secalins were rapidly degraded, at the beginning of the fermentations (Figure 5). In the sourdough prepared with the rye meal, which had a higher amylase activity and ash-content (RF-Ak), the proteolysis was more rapid. The proteolysis evidently also occurred when the RF-Ak was incubated at pH 3.6 in the absence of starter microbes, but no obvious secalin degradation occurred at pH 6.1.

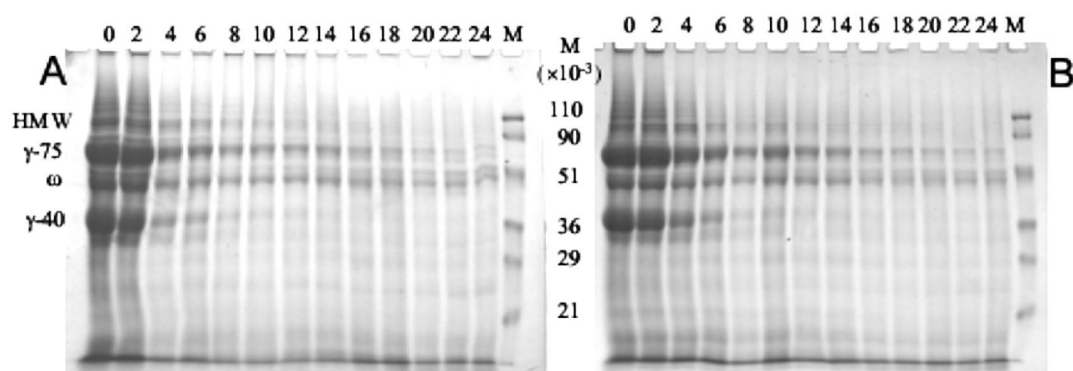


Figure 5. The hydrolysis of secalins during two RSD fermentations. SDS-PAGE gels A and B were loaded with alcohol-soluble extracts of samples collected from two rye sourdoughs made with RF-Ak and RF-Am ryes, respectively.

Kratochvil and Holas (1984) found similar results regarding proteolysis in rye sourdoughs when they followed the formation of protein hydrolysis products (amino acids and peptides) in the presence and absence of sourdough microbes. In addition, they showed that the proteolysis gradually intensified when the fermentation temperature was raised from 25°C to 40°C. This was partially due to the inhibited yeast growth that occurred at elevated temperatures and that prevented the yeast from consuming the hydrolysis products. After a confirmative study by Kratochvil and Holas (1988) and the work of Spicher and Nierle (1988), which revealed that there was a strain-specific accumulation of amino acids in rye sourdoughs, no further studies that dealt with the proteolytic phenomena that occur in rye sourdough were published until this study.

Rye is generally used as a whole grain in baking so that doughs containing it will include the proteolytic enzymes of the bran fractions; rye also usually has more proteolytic activity than

wheat (Table 6). The inhibitor studies described in this thesis showed that the aspartic proteinases were the dominant proteases in both of the tested rye cultivars, as more than 50% of the activity was arrested by the presence of pepstatin A. Substantial serine proteinase activity also existed (16-21% inhibition by PMSF). These results were nearly identical with those obtained in a previous study with rye bran proteases (Brijs et al 1999). Brijs et al (1999) also showed that the rye bran proteases hydrolysed gluten proteins and secalins *in vitro*.

Technological aspect: Sourdough proteolysis in rye baking

In rye baking the role of proteins is different than in wheat baking. Unlike gluten proteins of wheat the secalins are unable to develop gluten-like structures. The soluble rye proteins may, however, play another role in the formation of dough structures as they are involved in foaming (Meuser et al 2001). Such protein foams probably are formed during mixing of rye doughs and the foam structure could give continuity for the rye doughs and could play also a role in bread structure. During sourdough fermentations the acidification and proteolysis that occur increase the solubility of the secalins and produce soluble protein hydrolysis products and these may both enhance the formation of foam structures. These functional features of rye proteins (the inability to form gluten but the capacity to form foams) probably has traditionally encouraged the use of rye in sourdough baking.

4.1.3 Prolamin hydrolysis in germinated-wheat sourdoughs (III)

The hypothesis that extensive prolamin hydrolysis occurs in germinated-wheat sourdoughs (GWSD) was premised on two features:

- (1) The raw material containing high and diverse proteolytic activity would elicit also the hydrolysis of the gliadins that were only limited degraded in traditional wheat sourdoughs (I).
- (2) The pH-dynamic sourdough process would enable the sequential activation of diverse proteases having different pH-optima.

To test these premises, a germinated-wheat sourdough (GWSD) was prepared from germinated wheat grains that had high levels of diverse proteolytic activities. The cysteine proteinases were the dominant ones in these wheat grains, but other proteases were also

present, including proline-specific peptidase activities (POP, DPP IV). A single strain of heterofermentative *Lactobacillus brevis* that had a good acidifying capacity was used for the acidification.

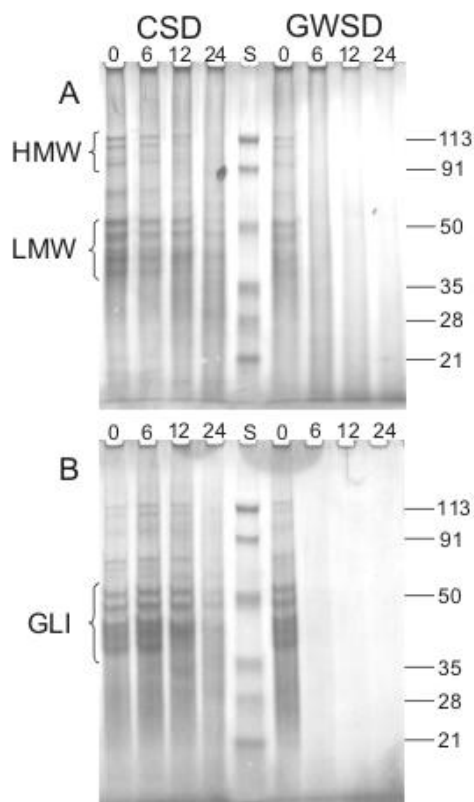


Figure 6. Gluten protein hydrolysis in a control sourdough (CSD) and a germinated-wheat sourdough (GWSD). SDS-PAGE gels A and B show the SDS-soluble and the alcohol-soluble proteins, respectively. The fermentation times are listed above the gels. The areas to which HMW and LMW glutenins and gliadins (GLI) migrated are indicated on the left sides of the gels, and the molecular weights (kDa) of standard proteins are listed on the right sides of the gels.

A rapid and extensive proteolysis occurred during the GWSD fermentation. Its free amino nitrogen (FAN) values were extremely high (FAN>1200 mg/kg) compared to those (FAN~220 mg/kg) of the control sourdough (CSD), which was prepared from ungerminated wheat. In addition, nearly all of the glutenins and gliadins were hydrolysed already during the first six hours of fermentation, after which they were not detectable with SDS-PAGE (Figure 6). Further evaluation of the extent of prolamin hydrolysis (that was carried out with an ELISA analysis) showed that during the GWSD fermentation the total prolamin contents diminished and less than 5% of the initial prolamins were detectable in the end of the

fermentation. The remaining prolamin content in the end of CSD fermentation was 64% of that in the beginning of CSD fermentation (Figure 7). In an ISD (inactive sourdough-like ferment prepared with heat-treated wheat) no hydrolysis of proteins occurred. These findings verify that the cereal proteases are responsible for the proteolysis that occurs in sourdough fermentations. It also is very evident that the protein degradation in sourdoughs is strongly related to the proteolytic activities of the used cereal materials.

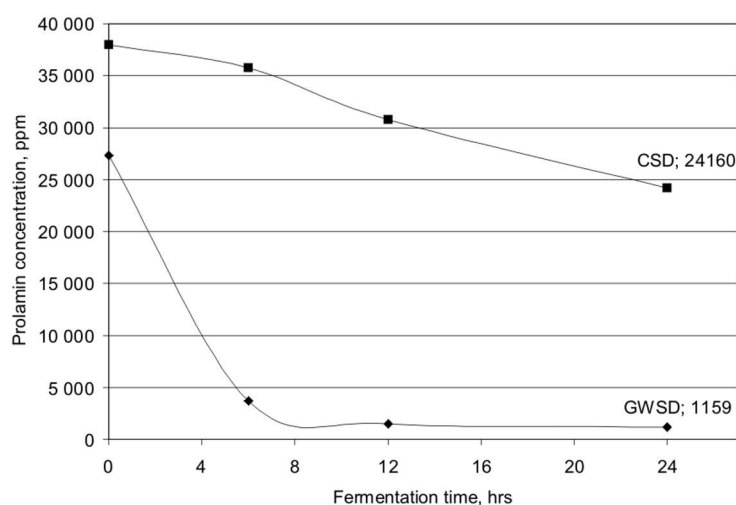


Figure 7. The prolamin concentrations of GWSD and CSD fermentations, as determined using a commercial ELISA procedure (Transia Plate *Prolamins*, Raisio Diagnostics, Finland).

The results from the GWSD fermentation are very promising. This study shows how traditional processing methods can be altered to provide novel applications. In this example germinated grains containing high proteolytic activities provide food technologists with an efficient method for exploiting natural and safe enzymes during food processing to develop healthy cereal-based products. It is also reasonable to expect that the natural proteolytic enzymes will hydrolyse their natural substrates efficiently.

In sourdoughs, the pH gradually declines during fermentation and this enables the different proteases, with their diverse pH-optima, to operate (see Table 2). The neutral conditions that predominate during the beginning of the sourdough fermentation favour the activities of the neutral proteases such as the serine and metalloproteinases of cereals. As the acidification proceeds the carboxypeptidases and aspartic proteinases become active. The pH-range of

sourdoughs is nearly ideal for the cysteine proteinases, which presumably were active throughout the GWSD fermentation. The wheat cysteine proteinases are also known to hydrolyse the gliadins efficiently (Dunaevsky et al 1989, Bottari et al 1996, Capocchi et al 2000), whereas the aspartic proteinases mainly prefer the glutenins as substrates (Kawamura and Yonezawa 1982, Bleukx et al 1998b). Thus, it is understandable that an effective hydrolysis of the gliadins occurred in the GWSD that was prepared from germinated wheat that had a high cysteine proteinase activity.

An interesting recent finding was that the pH-optimum for a recombinant barley cysteine proteinase (EP-B2) would be at a neutral pH (Bethune et al 2006). This result is contrary to the numerous results reported previously for natural barley cysteine proteinases (for a review, see Jones 2005). It thus seems that the recombinant EP-B2 that was expressed in *E. coli* differs from the natural barley proteinases by its pH-profile and also in its specificity (Zhang and Jones 1996, Davy et al 1998). Nevertheless, the study of Bethune et al (2006) suggested that the conversion of proEP-B2 (proenzyme) into EP-B2 (enzyme itself) occurred optimally under acidic conditions, but that the activity of the enzyme itself against a synthetic substrate was higher at neutral conditions. The study, however, also showed that the EP-B2 hydrolysed an α -gliadin at pH 4.5 and the 33-mer at pH 3.0 (no pH-values higher than 4.5 were tested in these experiments). Assuming that the suggestions of Bethune et al, however, are true, it would be worth trying to preincubate meals derived from germinated grains under acidic conditions and then to elevate the pH to neutral conditions before starting the acidification process. This, in theory, would allow any incompletely converted proEP-B2 to be changed into EP-B2 during the preincubation under the acidic conditions. Obviously, during the preincubation proteolysis, at least by aspartic proteinases, carboxypeptidases and the activated cysteine proteinases, would still be taking place. This initial proteolysis would produce prolamins hydrolysis products that could undergo further degradation. Raising the pH could then result in an enhanced activity of the cysteine proteinases and of the neutral proteases, including the metalloproteinases that have been shown, in barley mashing, to be active at pH 6.5. The neutral peptidases (Table 2), including the proline specific peptidases, would also become active and could take part in the gliadin hydrolysis.

Technological aspect: Sourdough proteolysis and celiac baking

In principal, the use of sourdoughs containing high proteolytic activity in baking technology that manufactures breads for celiac patients (celiac baking) could diversify the diets of celiac

patients by providing the healthy substances present in whole grains in their diets. Prior to recommending such baking technologies, however, trustworthy evidence of the detoxification of the prolamins is extremely important. In addition, it is important to follow the FAO/WHO guidelines for “gluten-free” products (Codex Alimentarius Commission 2004) that set the limitation for the gluten level not to exceed 200 ppm. In the Study **III**, the prolamins content in the end of the fermentation was ~1200 ppm, which means that the theoretical proportion of sourdough in baking should be ~15% at the maximum, requiring that the other ingredients are gluten-free.

A very recent study of Hartmann et al (2006) indicates that proteases of germinating grains can rapidly hydrolyse gliadin peptides into fragments that no longer are toxic for celiac patients. This recent result together with our findings of prolamins hydrolysis in GWSD fermentation, predict that the endogenous protease pool that is present in germinating cereals could be used in food processing in order to detoxify the celiac-toxic prolamins. Sourdough fermentation probably is one of the most potential food processes to conduct such extensive proteolysis, since it is a pH-dynamic semi-liquid food process that allows the activation of enzymes with different pH-optima.

It is clear that the extensive hydrolysis of cereal prolamins also imposes certain technological requirements on the baking, since it will obviously not be possible to produce a gluten based dough if practically all of the gluten proteins have been degraded (this apparently was the case in GWSD). In addition, the starch based formulas that are often used in celiac baking would also be problematic since the germinated grains will also contain high levels of amylolytic enzymes that might be partially active despite the low pH of the ‘dough’. One other possibility is thus to inactivate the amylases and then prepare a starch-based dough by using celiac-safe ingredients.

In fact, the use of germinated rye for celiac baking might be more feasible (than using wheat) since the formation of any gluten is irrelevant during rye baking. As mentioned earlier, the sourdough fermentation could actually increase the levels of soluble rye proteins, which may enhance the foam formation in rye doughs and may thus improve the structural properties of bread. Thus, for rye baking the degradation of the prolamins will not be that damaging to the bread structure. This seemingly makes germinated rye a potentially acceptable raw material for celiac baking in the future. Germinated rye also has a high level of diverse proteolytic enzymes, of which the most active are the cysteine and aspartic proteinases that both operate

under acidic conditions (Brijs et al 2002, Jones and Lookhart 2005). Such a baking technique, using germinated rye, would add the healthy substances found in rye to celiac patients' diets, and would also add flavour to the products.

4.2 Behaviour of oat 12 S globulins in oat bran fermentation (IV)

Oat bran fermentation (OBF) is used to produce non-dairy cereal products that have some similarities to yogurt. Typically such products have a jelly-like structure, a smooth taste and they are rich in dietary fibre and probiotic bacteria. In addition to its high dietary fibre content, oat bran is also rich in proteins, especially the 12 S globulins. We were the first to study the behaviour of these salt-soluble oat storage proteins in a pH-dynamic lactic acid fermentation. The objective of the study was principally to gather information about the protein changes that occur during OBF, while our specific interest was in the proteolytic phenomena and the solubilities of the oat proteins. In this section the main results of Study **IV** are examined and a hypothetic model for the behaviour of oat 12 S globulins in OBF is suggested.

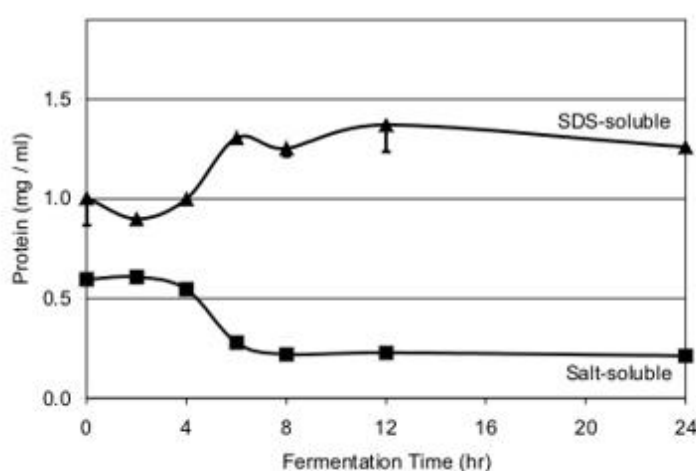


Figure 8. The shifts in the protein compositions of an oat bran fermentation. Apparently, most of the salt-soluble proteins changed into SDS-soluble proteins, but no proteolysis occurred.

No protein hydrolysis was observable during OBF, which is in contrast to the extensive proteolysis that is observable in sourdoughs. The lack of proteolysis in OBF likely was due to the absence of cereal proteases in the system. Instead of being proteolytically broken down, the oat 12 S globulins underwent solubility changes during OBF as was evident by the fact that they shifted from salt-solution to SDS-solution (Figure 8). Apparently this shift was induced by the change to a lower pH, which was confirmed by an oat globulin-solubility assay. It demonstrated that the salt-solubility of isolated globulins vanished under acidic pH-conditions.

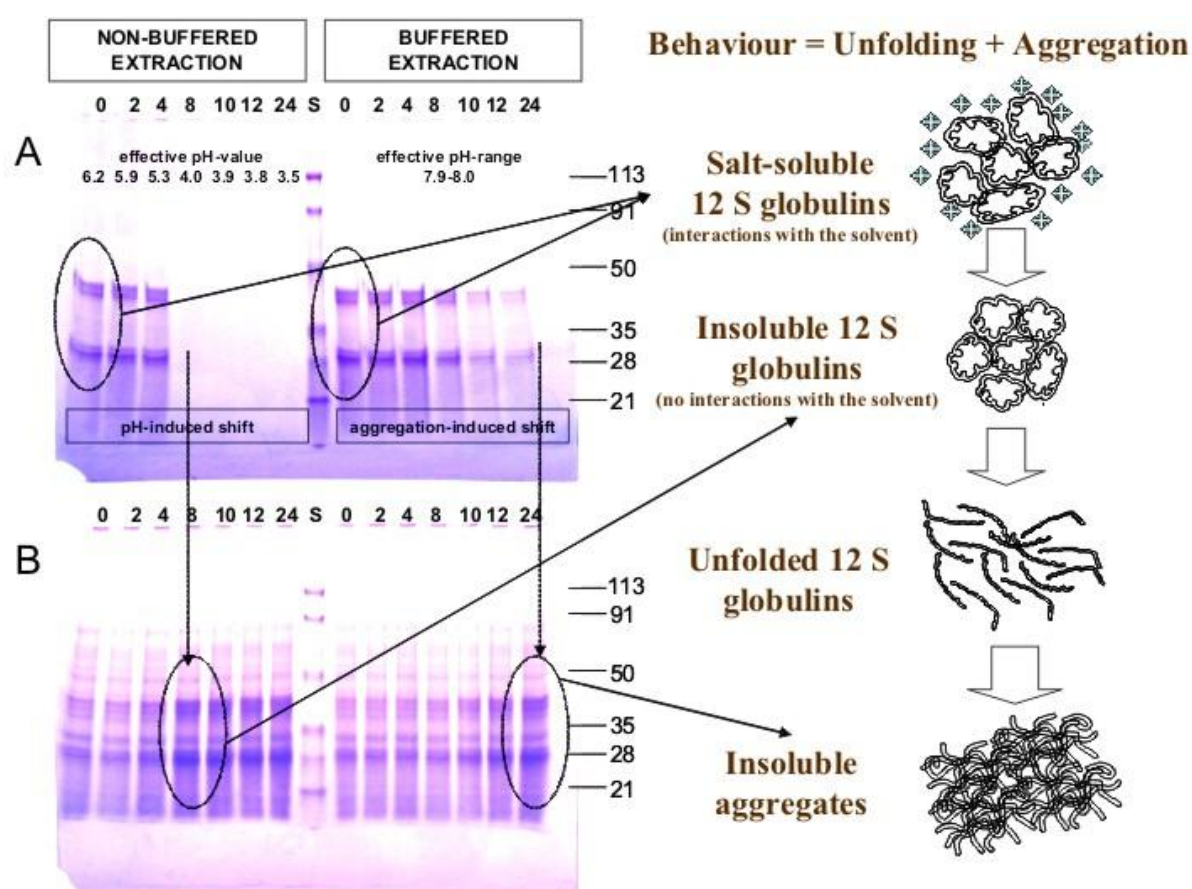


Figure 9. An illustration of the behaviour of oat 12 S globulins during an OBF. On the left, the A and B SDS-PAGE gels contain the salt-soluble and SDS-soluble oat proteins, respectively. The samples loaded onto the left side of gel A were extracted with non-buffered 1 M NaCl, whereas the samples on the right were extracted with 1 M NaCl buffered to pH 8. On the right is a hypothetical interpretation of the unfolding and aggregation of the oat 12 S globulins that occurred during acidification.

An illustration suggesting how the oat 12 S globulins might behave during OBF is presented in Figure 9. The left side of the upper gel presents OBF samples that were extracted with 1 M NaCl at the *in situ* pH, whereas the right side samples were extracted at pH 8. The lower gel contains the reductive SDS-soluble proteins (residual proteins) of the respective samples. The salt-solubility of the globulins evidently collapsed as the pH in OBF decreased from 5.3 to 4.0, which probably was due to the diminished repulsion forces between the proteins and the solvent at this pH-range. Generally, in the isoelectric pH-range the charge of a protein is low and the repulsion forces between the protein and the solvent weaken and therefore its solubility decreases. The isoelectric ranges of the acidic and basic subunits of the oat 12 S globulins are 4-5 and 7-8, respectively (Walburg and Larkins 1983). Apparently, in our particular system the interactions between the oat globulins and the solvent diminished between pH 5.3 and 4.0, as the solubility collapsed at this pH-range. In the globulin solubility assay, however, the isolated oat globulins became insoluble between pH 5-6. It is thus obvious that the processing history and chemical environment affects the solubility properties of oat globulins. Nevertheless, at this stage (8 hr samples) the 12 S globulins still dissolved in the buffered extract at pH 8. The observed shift in the globulin solubility was thus purely induced by the pH change.

Another type of solubility shift, however, was evident at the end of the fermentation when the globulins were even insoluble at pH 8 (Figure 9, right-side). This may be was due to an aggregation-induced protein shift that could have occurred. As concluded by Ma et al (2000), a decrease in pH may induce a mild denaturation of globulins. This means that they have been practically unfolded. The unfolded globulins are open-structured, so in time they probably interact with each other to form insoluble aggregates. Thus, two behavioural features of the oat 12 S globulin may be distinguished during the OBF: the pH-induced unfolding and a further aggregation of the unfolded proteins (Figure 9).

Thus, briefly, the preparation of OBF begins with the boiling of the oat bran and water. The oat globulins tolerate this heat-treatment well and no changes in the solubility of the 12 S protein occur; the oat globulins are very heat-stable proteins and readily tolerate boiling temperatures (Ma and Harwalkar 1987, Marcone et al 1998). However, despite their good tolerance to boiling, the oat globulins are sensitive to acidification. Acidification leads to the unfolding and subsequent aggregation of oat 12 S globulins (Figure 9).

Similar phenomena occur, on purpose, during the acidification of heat-treated milk proteins. During heating, the milk proteins denature, whereas acidification induces their aggregation that leads to the formation of gel-like structures. In OBF such protein aggregation, however, apparently has no major effect on the structural properties of the product, since the gelatinised starch and non-starch polysaccharides are the main determinants of its structure (Jaskari et al 1996).

Theoretical aspect: Production of ACE-inhibitory peptides

The negligible role that the oat globulins play in the product structure, in principal, allows their modification by proteolytic enzymes, since their degradation apparently causes no structural changes. The absence of interfering proteases also enables the proteolysis that does occur to be targeted in order to produce the exact hydrolysis products (i.e. the amino acids or peptides) that are wanted. The production of such specific hydrolysis products could, for instance, be directed toward specifically providing flavour precursors or antifungal compound precursors, or even produce functional peptides that have enhanced health effects.

Among such peptides are the blood-pressure lowering peptides that are already used by the dairy industry (Seppo et al 2003). The effectiveness of these is based on their ability to inhibit a blood-pressure regulator called angiotensin-converting enzyme (ACE). Several ACE-inhibitory peptides have been isolated from food proteins and characterised, and most of these apparently occur in the storage proteins of wheat, barley, rye, and oats, including the oat 12 S globulins (Table 7) (Loponen 2004). The ACE-inhibitory tripeptides that occur in the cereal proteins often have a branched chain amino acid (Leu, Ile, Val) at their N-terminal ends and proline at the C-terminus (Loponen 2004). The proteolytic liberation of such peptides by targeted proteolysis is possible, but will require specific proteolytic enzymes. In theory, a sequential hydrolysis with thermolysin (EC 3.4.24.27) and proline oligopeptidase (EC 3.4.21.26) could liberate such tripeptides from several cereal protein structures. In the first place, a proteinase (thermolysin) is needed that can initiate the hydrolysis of intact proteins, whereas the second phase requires specific peptidases acting on the previously formed oligopeptides. Thermolysin preferably cleaves a peptide bond C-terminal to leucine (Xcc-↓-Leu) but in a rye secalin hydrolysis it also frequently cleaved peptide bonds occurring C-terminal to isoleucine and valine (Gellrich et al 2004). A hydrolysis with thermolysin thus seems to produce oligopeptides that have a branched chain amino acid at their N-termini. A subsequent cleavage of such an oligopeptide with a POP-type enzyme would thus produce

peptides with proline as their C-terminal amino acid, and such a sequential hydrolysis could thereby liberate ACE-inhibitory peptides from cereal storage proteins. Interestingly, the POP-type enzymes also play a key role in the detoxification of gluten peptides (see 2.3.2).

The OBF thus differs drastically from sourdoughs, in that it lacks the cereal enzymes and thus no proteolysis occurs during the fermentation. This inactive nature of OBF enables various possibilities for approaching the system, including using proteolysis, as indicated above. The OBF, together with other proteolytically inactive fermentations, offers a rather new research field for many scientists. For instance, LAB researchers can test their strains in cereal fermentations in order to look for proteolytic strains that can be used for food processing. In addition to proteolytic approaches, numerous other features are to be studied in order to develop innovative new cereal fermentation techniques.

Table 7. The occurrence of known ACE-inhibitory tripeptide amino acid sequences in the storage proteins of wheat, rye, barley and oats. The list is adapted from Loponen (2004), which also includes complete reference information.

Seq	Occurrence	References
DLP	γ -secalins, LMW glutenins	Wu and Ding 2002
GGY	LMW glutenins	Saito et al 1994
GPV	oat 12 S globulins , HMW glutenins	Kim et al 2001
HHL	γ -secalins	Shin et al 2001
IPP	LMW glutenins, α -gliadins	Nakamura and Yamamoto 1995
IRA	B-hordeins, LMW glutenins, γ -gliadins	Miyoshi et al 1991
IVY	B-hordeins, LMW glutenins	Matsui et al 1999
LKP	oat 12 S globulins	Fujita and Yoshikawa 1999
LPP	LMW glutenins, γ -gliadins	Yamamoto 1997
LQP	avenins, B, C, D, γ -hordeins, HMW secalins, ω -secalins, HMW glutenins, LMW glutenins, α , γ , ω -gliadins,	Miyoshi et al 1991
LRP	γ -hordeins, γ -gliadins	Miyoshi et al 1991
LSP	oat 12 S globulins	Miyoshi et al 1991
PRY	HMW secalins, HMW glutenins	Saito et al 1994
VPP	avenins, γ -hordeins, HMW secalins, HMW glutenins, γ -gliadins	Nakamura and Yamamoto 1995
VRP	γ -gliadins	Matsumura et al 1993
VSP	D-hordeins, HMW glutenins	Miyoshi et al 1991

5 CONCLUSIONS

The degradation of prolamins was evident in all sourdough types and the proteolytic activity originated from cereals. This is logical since the cereal proteases typically are active under the acidic conditions that are present in sourdoughs. Of the cereal proteases, the aspartic proteinases were the predominant ones in wheat sourdoughs (WSD, **I**) and in rye sourdoughs (RSD, **II**) whereas the cysteine proteinases dominated in the germinated-wheat sourdough (GWSD, **III**) that was prepared from germinated wheat grains. In another type cereal fermentation system, oat bran fermentation (OBF, **IV**), no protein degradation was observed, which was due to the absence of any active cereal proteases in this fermentation.

In WSD, the polymeric HMW glutenins underwent depolymerisation prior to their proteolytic degradation. The depolymerisation reduces the polymer size and increases the solubility of HMW glutenins, which presumably renders them more susceptible for proteolytic breakdown. Gliadins were not degraded extensively in in this system. Compared to WSD, the degradation of secalins in RSD was rapid and extensive, which is explainable by the higher proteolytic activity of rye. Similar proteolytic hydrolyses occurred in the wheat and rye fermentations in the absence of starter microbes when meal suspensions were incubated under acidic conditions. In GWSD, the hydrolysis of the wheat prolamins (glutenins and gliadins) was rapid and extensive, which was due to the highly proteolytic meals (from germinated wheat) that were used as a raw material in the fermentation. The prolamins hydrolysis was very extensive and such processing, after optimisation, could offer new insights into baking methods for celiac patients.

This thesis confirms that the cereal proteases degrade cereal prolamins in sourdoughs, and that the extent of hydrolysis is related to the proteolytic activity of the cereal raw materials that were used. Obviously, however, the lactic acid bacteria play also a key role in the sourdough proteolysis by producing acids that lower the sourdough pH. These acidic conditions are required for the cereal proteases to be active and thus for the proteolytic degradation of the prolamins in sourdoughs.

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